

CITRUS VARIEGATED CHLOROSIS: DEVELOPMENT OF TRANSGENIC RESISTANCE
AND MOLECULAR STUDIES OF PATHOGENESIS

By

RICARDO HARAKAVA

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY
OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2000

To my parents Kyoko and Gensei Harakava,

For helping me to accomplish my dreams

ACKNOWLEDGEMENTS

I would like to express my gratitude to my advisors, Dr. Richard F. Lee and Dr. Dean W. Gabriel, for their guidance, unconditional support, and constant encouragement before and during my graduate studies. I am also grateful to the members of my committee, Dr. Charles L. Niblett, Dr. Ken S. Derrick, Dr. Charles Powell, and Dr. Gloria A. Moore, for reviewing the manuscript and enriching my research with their insights.

I am very grateful to Chandrika and Manjunath for their kind and unconditional support.

I thank my friends Abdulwahid, Adriana, Asha, Basma, Bayram, Blanca, Denise, Eduardo, Francisco, Gary, Gustavo, Hanife, Jorge, Juliana, Marlene, Mehtap, Ping, Simone, Ufuk, Vicente, Yolanda.

I thank my friends at the Instituto Biológico, with a very special mention to Julia Beretta and Elza Martins.

I thank my friends at the Brazilian Student Association (BRASA).

I thank Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP), for providing the scholarship that allowed me to accomplish this work.

I thank Fundo Paulista de Defesa da Citricultura (FUNDECITRUS), for providing the financial support to my research.

TABLE OF CONTENTS

	<u>Page</u>
ACKNOWLEDGEMENTS	iii
ABSTRACT	v
CHAPTERS	
1 INTRODUCTION	1
<i>Xylella fastidiosa</i> and Citrus Variegated Chlorosis	1
Transgenic Resistance Against Bacterial Plant Pathogens	6
Xylem-specific Gene Expression	12
Genetic Comparison Between Bacterial Strains	14
Objectives	15
2 INOCULATION OF TOBACCO PLANTS WITH <i>X. fastidiosa</i>	16
3 CLONING OF A PAL PROMOTER FROM SWEET ORANGE	19
4 TRANSFORMATION OF TOBACCO PLANTS WITH THE GUS GENE UNDER CONTROL OF THE CITRUS PAL PROMOTER	31
5 SYNTHESIS OF A CECROPIN GENE AND EXPRESSION IN TRANSGENIC TOBACCO UNDER CONTROL OF THE CITRUS PAL PROMOTER	39
6 COMPARISON BETWEEN CVC AND PD STRAINS OF <i>X. fastidiosa</i> THROUGH SUPPRESSION SUBTRACTIVE HYBRIDIZATION	53
7 CONCLUSIONS	63
REFERENCES	65
BIOGRAPHICAL SKETCH	71

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

CITRUS VARIEGATED CHLOROSIS: DEVELOPMENT OF TRANSGENIC RESISTANCE
AND MOLECULAR STUDIES OF PATHOGENESIS

By

Ricardo Harakava

August 2000

Chair: Richard F. Lee

Cochair: Dean W. Gabriel

Major Department: Plant Pathology

Xylella fastidiosa is a xylem-inhabiting, pathogenic bacterium that is spread by insect vectors and which causes severe diseases in many plants, including citrus, grape, coffee, plum, peach, and oak. Its localization in the xylem poses a challenge for control of this pathogen by any method, including the development of transgenic resistance. Mature xylem vessels are composed of dead cells that lack intrinsic metabolic activity, but are surrounded by living xylem parenchyma that remain active throughout the life of the plant. Xylem parenchyma shows high activity of the enzyme phenylalanine ammonia-lyase (PAL), which is required for the synthesis of phenylpropanoid precursors of the lignin that reinforces the walls of xylem vessels. The high level of expression and tissue specificity of the PAL gene make the PAL promoter a potentially useful candidate for development of transgenic plants that produce antimicrobial peptides in xylem tissues. Because *X. fastidiosa* causes a severe citrus disease, citrus variegated chlorosis (CVC), and there are no available chemical control methods, development of genetic resistance to CVC has become a high priority in many labs worldwide. Tobacco was demonstrated to be colonized by *X. fastidiosa*, and used as a model to study the effect of cecropin gene expression

against the infection by this pathogen. A PAL promoter was cloned from sweet orange through inverse-PCR and demonstrated to express the reporter protein GUS preferentially in the xylem of transgenic tobacco. A gene for the antibacterial peptide cecropin MB39, preceded by a signal peptide for export, was synthesized by PCR. Tobacco plants were transformed with a construct containing the cecropin gene under control of the sweet orange PAL promoter, and transgene expression was demonstrated.

Impairment of plant development was observed in some of the transgenic lines expressing the cecropin gene. The high level of transgene expression conferred by the sweet orange PAL promoter, with enhanced activity in the xylem, may have been the cause of this phenotypic alteration. Further investigations are necessary in order to determine the appropriateness of using lytic peptides to develop transgenic citrus resistant to bacterial pathogens.

CVC arose as an entirely new disease in 1987, and its origins are puzzling. In an attempt to determine what might account for the “new” host range of *X. fastidiosa*, CVC DNA was compared to a closely related strain of the species that causes Pierce’s Disease (PD) of grapevines. The comparison of CVC and PD strains of *X. fastidiosa* was performed using a suppression subtractive hybridization procedure. Sequencing of 18 clones revealed that the PD strain lacks a 51 kb plasmid present in the CVC strain, and that bacteriophage-related genes are responsible for some of the differences between the two strains.

CHAPTER 1 INTRODUCTION

Xylella fastidiosa and Citrus Variegated Chlorosis

Xylella fastidiosa is a gram negative, xylem-inhabiting fastidious bacterium that causes severe diseases in a wide range of plant species and also colonizes numerous others without showing obvious symptoms. It was first associated with Pierce's Disease (PD) of grapevines through electron microscopy observations, in the early seventies (Goheen *et al.*, 1973; Hopkins and Mollenhauer, 1973), and axenically cultured in 1978 (Davis *et al.*, 1978). Initially, PD was thought to be caused by a *Rickettsia*-like organism, due to the similarity of their cell envelope morphology. Also, the first successful isolation of the PD bacterium was done using a medium based on that used to culture the trench fever rickettsiae, *Rochalimaea quintana*. Other similarities included the growth preference of organic acids to dextrose and the transmission by an insect vector. However, after its isolation in artificial medium, the genetic dissimilarity between these two groups of bacteria became evident. The GC content calculated for different strains of *X. fastidiosa* varied from 49.5% to 51.7%, well above the value for *R. quintana* (38.5%) and all other rickettsiae (Kamper *et al.*, 1985). Ribosomal small subunit RNA (16SrRNA) gene sequencing revealed that *Xanthomonas* was the closest genus, within the gamma subgroup of the eubacteria (Wells *et al.*, 1987). The PD-causing organism was then described as fastidious xylem-limited bacteria or xylem-inhabiting bacterium, until Wells and colleagues (1987) proposed the new genus *Xylella* to accommodate this group of plant pathogens.

X. fastidiosa is mainly vectored by sharpshooter leafhoppers (Family Cicadellidae, subfamily Cicadellinae) but spittlebugs or froghoppers (Family Cercopidae) also can transmit this bacterium. After feeding on the xylem of an infected plant, these insects become immediately infective and remain so until they molt. The lining of the foregut, to which the bacterium adheres, is shed upon molting (Purcell and Finlay, 1979).

Besides PD other plant diseases that became known to be caused by *X. fastidiosa* are: alfalfa dwarf, phony peach, plum leaf scald, periwinkle wilt, almond leaf scald, oak leaf scorch, elm leaf scorch, sycamore leaf scorch, maple leaf scorch, ragweed stunt, pear leaf scorch, citrus variegated chlorosis (CVC), coffee leaf scorch, and oleander leaf scorch (Hopkins, 1989; Purcell and Hopkins, 1996). Several riparian plant species are also systemically infected by *X. fastidiosa* and are important components in the epidemiology of PD in California (Purcell and Saunders, 1999).

Although molecular and biochemical data indicate that all strains of *X. fastidiosa* are similar enough to be considered as a single species, differences in host range and nutritional fastidiousness do exist. Host range experiments are particularly difficult to conduct with *X. fastidiosa* strains due to the long incubation time required for symptoms to appear and because most of the hosts are perennial plants. It is cumbersome for a single laboratory to have a whole set of them available for inoculations. Reciprocal transmission tests indicate that PD, almond leaf scorch, and alfalfa dwarf are caused by the same strains of *X. fastidiosa*, and that phony peach and plum leaf scald are caused by the same strains, but the two groups of diseases are caused by different strains (Davis *et al.*, 1980; Wells *et al.*, 1981b). The plum leaf scald and phony peach strains seem to be nutritionally more fastidious than the PD strain, since the former grow only on PW medium, while the latter grow on any media developed for *X. fastidiosa* (Davis *et al.*, 1981; Wells *et al.*, 1981a). It has been shown that PD strains can grow even on nutrient agar medium, but at slower rates when compared to specialized media (Chang *et al.*, 1990; Fry *et al.*, 1990). Comparison of the genomes of these strains using restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) support these data by showing strong

similarity between PD, almond leaf scorch, and alfalfa dwarf strains (Chen *et al.*, 1992; Pooler and Hartung, 1995). These molecular studies also revealed similarity between plum leaf scald and elm leaf scorch which form a group distinct from the PD group. Mulberry leaf scorch strains are closer to PD strains and periwinkle wilt strains are closer to the plum and elm strains. CVC strains are only distantly related to other *X. fastidiosa* strains (Pooler and Hartung, 1995). This result is not surprising given the South American origin of the CVC strains, as opposed to the North American origin of all the other strains. The existence of genetic divergence between the strains from these two geographic regions is further supported by the results of arbitrarily primed-PCR (Costa *et al.*, 2000).

Studies of the genetic relatedness among *X. fastidiosa* strains are relevant for understanding the evolution and host specialization of this pathogen but, in certain cases, they can be misleading. When the coffee leaf scorch strains were discovered, they were thought to be identical to the CVC strains, since primers that were developed specifically for the citrus isolates could amplify identical PCR products from the coffee strains, and RAPD-PCR studies could not distinguish between the two of them (Lima *et al.*, 1998; Rosato *et al.*, 1998). Nevertheless, reciprocal transmission experiments show that these strains are host-specific, indicating that adaptation to a different host may not require major genetic changes.

Of particular interest to the present study is the disease caused by *X. fastidiosa* on citrus trees. CVC was first detected in groves in the northwest region of São Paulo State, Brazil, in 1987. Although initially suspected to be a zinc nutritional deficiency, it was soon shown by electron microscopy that a bacterium morphologically similar to *X. fastidiosa* was associated with the disease (Chagas *et al.*, 1992). Through serological assays employing polyclonal antisera made against a PD strain of *X. fastidiosa*, it was demonstrated that symptomatic plants always resulted in positive reactions (Lee *et al.*, 1993). Koch's postulates were fulfilled in 1993, eliminating any remaining doubt about *X. fastidiosa* being the cause of CVC (Chang *et al.*, 1993; Lee *et al.*, 1993). In just a few years the disease was found widespread throughout São Paulo State and all

other citrus producing areas in Brazil. Contaminated nursery plants are probably to blame for the rapid dissemination, with the help of a diverse population of sharpshooter leafhoppers spreading the pathogen locally once an infected plant was introduced in a grove. Symptoms of the disease are interveinal leaf chlorosis, gummy brown lesions on the abaxial face of the leaf, reduced fruit size accompanied by hardened rind, and general thinning and wilting of the canopy. Sweet orange is the most severely affected variety, independent of the rootstock, while most of the mandarins, limes, lemons, grapefruits, and several interspecific hybrids either show mild symptoms or no symptoms at all (Laranjeira *et al.*, 1998). Trees less than seven years of age are more severely affected by CVC.

Surveys conducted by the Fund for Citrus Plant Protection (FUNDECITRUS), in 1994, 1996, and 1997, indicated disease incidence values of 16.9%, 23.7%, and 34%, respectively, for CVC in São Paulo State and south of Minas Gerais State (<http://www.fundecitrus.com.br>). The percentage of trees in an advanced stage of the disease (symptoms throughout the canopy and production of small fruits) was 4.7%, 6.8%, and 10.4% for those three years, respectively. Considering that the total number of sweet orange trees in this region was 184.8 million in 1998, it was estimated that around 19.2 million of these would be rendered nonproductive due to CVC.

Citrus growers realized that CVC could be controlled in adult trees by pruning affected branches 0.5 to 1.0 m below the lowest symptomatic leaf, when the initial symptoms occurred (<http://www.fundecitrus.com.br/cvc.html>). Trees that were two years old and younger, when symptomatic, had little chance of recovery by pruning and had to be eradicated. Control of the sharpshooter population, through frequent application of insecticides and control of the spontaneous vegetation was also necessary. The theoretical basis for this control method is that *X. fastidiosa*, after being inoculated by a vector into a young flush, moves relatively slowly downward through the vessels of a branch. An important factor in the success of this method is that spread of CVC in the grove occurs from citrus to citrus with no alternative weed host present. Alternative hosts in the native vegetation apparently do not play an important role in the

disease epidemiology. In contrast, pruning cannot control PD in Florida, since the inoculum is commonly found in wild grapevine relatives and other plants of the natural vegetation, and efficient leafhopper vectors are present. This fact limits Florida's viticulture to muscadine vine species, which are resistant or tolerant to PD. In California, cultivation of European grapevine varieties has been possible because normal pruning practices and colder winters, with the associated physiological changes they bring to the host, eliminate most of the bacteria in infected cultivated vines. Re-infection occurs via leafhoppers that acquire the bacteria from riparian plants where both the vector and the pathogen over-winter. This cycle results in a linear progression of the disease, with limited damage to vineyards. The recent establishment of the glassy-winged sharpshooter (*Homalodisca coagulata*) in California may change this picture (<http://www.cnr.berkeley.edu/xylella/>). This vector, which is commonly found in the Southeastern US, regularly feeds and reproduces on grapevines, appears to move faster and longer than other sharpshooters already present in California, can utilize a wide range of plants and habitats for feeding and breeding, and is often found feeding on older stems of vines where *X. fastidiosa* has a better chance of surviving the following winter and to escape pruning, ultimately leading to a chronic infection of the host and an exponential increase of the disease in the vineyard.

The impact of CVC on the citrus industry in São Paulo State, allied to the need to stimulate research in the area of molecular genetics, prompted the state agency responsible for funding scientific research (Fundação de Amparo a Pesquisa do Estado de São Paulo – FAPESP) to implement the *X. fastidiosa* genome sequencing project. With a budget of US\$ 15 million from FAPESP and FUNDECITRUS, a network of 35 sequencing laboratories throughout São Paulo State was created in October of 1997. By January 2000, the entire genome of a CVC strain of *X. fastidiosa* (2,678,960 bases) was sequenced and assembled. This is the first plant pathogenic bacterium to be sequenced in the world. Based on the data generated, various functional genomics

projects were started aiming to clarify the mechanisms of pathogenicity and new ways to control CVC.

Transgenic Resistance Against Bacterial Plant Pathogens

Chemical control of bacterial plant diseases is, in most cases, limited to protective applications of copper products. The use of antibiotics, like streptomycin or tetracycline, is either economically unviable or lead to the appearance of resistant strains of the pathogens. Other methods for control of bacterial diseases are clean cultural practices, disease eradication, and/or, when available, natural host resistance. Of the alternatives available, the use of natural host resistance is the most desirable, but breeding of perennial crops is severely impaired by the lengthy life cycles of these plant species. Furthermore, for many species, including most citrus varieties, traditional breeding is complicated by the high heterozygosity of stocks, pollen and ovule sterility, sexual incompatibility, and widespread occurrence of nucellar embryony (Grosser and Gmitter, 1990). Given this scenario, the necessity of finding alternative ways for the genetic improvement of perennial crops becomes evident.

The use of somatic hybridization through protoplast fusion constitutes a potentially useful tool for the introduction of new traits, and its application for the improvement of citrus varieties has been extensively reviewed (Grosser and Gmitter, 1990). Generation of allotetraploids with additive features from each of the parent lines and circumvention of sexual incompatibilities are some of the possibilities of this technology. However, its usefulness for the development of sweet orange lines resistant to *X. fastidiosa* is uncertain since genetic resistance to this pathogen seems nonexistent within the species, and the nature of the tolerance/resistance in close relatives is poorly understood.

Development of disease resistance through genetic engineering is particularly attractive for the improvement of perennial crops. It can represent a shortcut avoiding decades of traditional

breeding or, even more interesting, it can make use of genes that are inaccessible by natural means. The use of transgenic resistance against plant viruses is in a more advanced stage than for microbial pathogens. This can probably be explained by our better understanding of the pathogenicity mechanisms employed by the viruses, and by the availability of gene silencing as a tool for blocking viral replication (Bent and Yu, 1999). In contrast, fungi and bacteria have more diversified, complex, and therefore less well understood mechanisms of pathogenicity, giving fewer immediate targets for engineering resistance.

Strategies that are being pursued for the development of transgenic resistance against prokaryotic plant pathogens include cloning and introduction of natural plant resistance genes, expression of exogenous degradative enzymes, expression of genes that cause breakdown of or insensitivity to toxins produced by the pathogen, expression of "plantibodies" against antigens from the pathogen, and expression of proteins that are inhibitory or lethal to the pathogen (Bent and Yu, 1999; During, 1996).

Several resistance genes that recognize bacterial plant pathogens have been cloned (Bent and Yu, 1999). The usefulness of these in the development of new resistant lines will depend upon the importance for the pathogen of the cognate avirulence gene that is the target of the resistance. Evidence indicates that many avirulence gene products have critical pathogenicity functions while others are gratuitous. The effect of mutations in these avirulence/pathogenicity genes will dictate the stability and effectiveness of the resistance genes that can recognize them (Gabriel, 1999).

Over-expression of resistance genes or components of the signal transduction cascade have been shown to confer broad-spectrum constitutive resistance through activation of systemic acquired resistance (SAR). Constitutive expression of the tomato *Pto* and *Prf* genes confer resistance not only to *P. syringae* pv. *tomato* but also to *X. campestris* pv. *vesicatoria*, *Ralstonia solanacearum*, *Cladosporium fulvum*, and tobacco mosaic virus (TMV) (Oldroyd and Staskawicz,

1998; Tang *et al.*, 1999). The unexpected absence of deleterious effects, which normally occurs when SAR is constitutively activated, makes this strategy worth pursuing in different crops.

Among the pathogenesis-related (PR) proteins endoglucanases and chitinases are being used for the development of transgenic resistance against fungal plant pathogens. Given the lack of knowledge about plant enzymes with degradative action against bacterial pathogens, molecular geneticists are left to the utilization of exogenous enzymes with such properties. Lysozymes from human and bacteriophage T4 sources have been used for the development of transgenic tobacco and potato with resistance to bacteria and/or fungi (During *et al.*, 1993; Nakajima *et al.*, 1997).

Plantibodies (antibodies or antigen binding fragments from these expressed in plants) have been used for the development of resistance against viruses. Targeting those molecules against viral components and expressing them in the plant cytoplasm can interfere with viral replication and lead to resistance to the virus. Application of this strategy to develop resistance against bacteria is less likely to succeed since binding of antibodies to the surface of the pathogen alone has little or no effect on its replication. Exceptions to this generalization are the wall-less prokaryotes, spiroplasmas and phytoplasmas. It has been demonstrated that a monoclonal antibody targeted to surface antigens of the corn stunt spiroplasma was able to inhibit its growth *in vitro* (Chen and Chen, 1998). The single-chain (Sc) Fv fragment gene derived from this monoclonal antibody was cloned and introduced into maize plants, but disease resistance was not attained in the transgenic lines. Possible explanations given by the authors for this failure are (1) reduced inhibitory activity of the ScFv antibodies as compared to the hybridoma-derived monoclonal antibodies, (2) ScFv antibodies may not have been transported to phloem sieve tubes where the pathogen resides, and (3) low levels of ScFv gene expression. Despite this negative result, the strategy is worth pursuing and is more likely to succeed once ScFv antibodies with enhanced affinity and stronger tissue-specific promoters are used.

Many phytopathogenic bacteria produce toxins which constitute primary determinants of disease by suppressing host resistance mechanisms. Therefore, transformation of plants with

genes encoding detoxifying enzymes has the potential to generate resistant lines. Indeed, Zhang *et al.* (1999) were able to develop transgenic sugarcane plants resistant to *Xanthomonas albilineans* through the introduction of a gene from the bacterium *Pantoea dispersa* which encodes an esterase which detoxifies allicidins (low molecular weight toxins from *X. albilineans*). By nullifying the effect of allicidins these authors enabled the host to manifest its natural resistance and block multiplication of the pathogen.

Bacterial resistant transgenic plants also have been developed by the expression of antimicrobial peptides from plants (alpha-thionin and lipid transfer proteins) and of non-plant origin (human lactoferrin and cationic antimicrobial peptides from insects) (Carmona *et al.*, 1993; Huang *et al.*, 1997; Molina and Garcia-Olmedo, 1997; Norelli *et al.*, 1999; Ohshima *et al.*, 1999; Reynoird *et al.*, 1999; Zhang *et al.*, 1999). Carmona *et al.* (1993) produced transgenic tobacco that expressed an alpha-thionin gene from barley. The gene product, a 5 kDa cysteine rich protein, has long been known to be toxic to plant pathogens, is found abundantly in cereal seeds endosperm and is stress-induced in leaves. The transgenic plants showed reduced leaf necrosis when infiltrated with suspensions of *P. syringae* pv. *tabaci* and *P. syringae* pv. *syringae*. Other proteins from barley that have been used because of their antimicrobial properties are the lipid transfer proteins (LTP). These proteins were named after their ability to stimulate the transfer of a broad range of lipids between membranes *in vitro*. Transgenic tobacco expressing a LTP gene showed a reduction in necrotic lesions caused by *P. syringae* pv. *tabaci* (Molina and Garcia-Olmedo, 1997). The same gene, when expressed in *Arabidopsis thaliana*, conferred the same reduction in symptoms caused by *P. syringae* pv. *tomato*.

Human lactoferrin is an iron-binding glycoprotein that plays an important role in infant defense systems. It has a potent bactericidal domain in the N-terminal region, called lactoferricin, which can be released by proteolytic cleavage. Expression of a human lactoferrin gene under control of CaMV 35S promoter in tobacco plants conferred delay of wilting symptoms after inoculation with *Ralstonia solanacearum* (Zhang *et al.*, 1998). Apparently, the full-length protein

was being cleaved by plant proteases to release the bactericidal lactoferricin fragment. Transgenic plants also showed a reduction of 10% in size, as compared to wild type plants, which could probably be explained by a reduction in iron availability due to the strong iron-binding ability of lactoferrins.

Cationic peptides constitute a new class of antimicrobial compound that has received a lot of attention in recent years in the medical area due to their potential use as a replacement or as a complement to traditional antibiotics (Hancock and Lehrer, 1998). These small peptides (12 to 45 amino acids) are structurally diverse but most are composed of an amphipathic α -helix (cecropins, magainins, sarcotoxins) or β -sheet elements stabilized by intramolecular cysteine disulphide bonds (mammalian defensins, thionins, tachyplesins). Their main site of action is the cytoplasmic membrane, where these peptides tend to assemble to form channels. The higher susceptibility of bacterial cells to these peptides results from the higher content of anionic lipids on the surface of the bacterial cytoplasmic membrane, the high electrical-potential gradient across this membrane (negative inside), and the absence of cholesterol molecules. In Gram-negative bacteria there is an initial interaction with the highly anionic outer-membrane lipopolysaccharide followed by a process called self-promoted uptake that gives the cationic peptides access to the cytoplasmic membrane. Most of these small peptides were isolated from insect hemolymph, where they play an important role in the defense against bacterial infections, but they also are present in higher animals and plants as well.

In vitro assays have shown that cecropins are highly toxic to several Gram-negative plant pathogenic bacteria (Hightower *et al.*, 1994; Nordeen *et al.*, 1992; Owens and Heutte, 1997). Although the methodology employed as well as the type of cecropin used varied from one reference to another, generally concentrations below 5 μ M were sufficient to inhibit growth of bacteria belonging to the following genera: *Agrobacterium*, *Clavibacter*, *Erwinia*, *Pseudomonas*, *Ralstonia*, and *Xanthomonas*.

With such high efficacy against bacterial plant pathogens, one would expect high resistance in transgenic plants expressing cecropin genes. However, this is not always the case, as Allefs *et al.* (1995) and Hightower *et al.* (1994) observed in their potato and tobacco transgenic plants, respectively, containing a cecropin B gene. These transgenics did not show the expected enhanced resistance against *Erwinia carotovora* subsp. *atroseptica* and *E. chrysanthemi* (in potato) or *P. syringae* pv. *tabaci* (in tobacco). mRNA corresponding to the transgene could be detected in both studies, but the concentration of the antibacterial peptide was very low (less than 0.0001% of total protein, as measured by indirect ELISA) or too low to be detected (through Western blot), most probably because of the action of plant proteases. To circumvent the action of proteases present in the intercellular fluid, Owens and Heutte (1997) developed a cecropin B analogue (MB39) that showed a half-life, on the average of 2.9 times greater than the original peptide when incubated with intercellular fluids from several plant species. Efficacy against several plant pathogenic bacteria was not significantly affected by the modifications introduced into MB39.

Despite these stability problems, several groups reported enhanced resistance in transgenic plants expressing different cationic antimicrobial peptides. Expression the modified cecropin MB39, under control of a proteinase inhibitor II wound-inducible promoter, conferred resistance in tobacco against *P. syringae* pv. *tabaci* (Huang *et al.*, 1997). A sarcotoxin IA gene expressed under control of a CaMV 35S promoter conferred resistance in tobacco against *P. syringae* pv. *tabaci* and *E. carotovora* subsp. *carotovora* (Ohshima *et al.*, 1999).

Attacin E is another protein that is part of the insect immune response and it has been used for the development of transgenic plants. Larger than the other cationic peptides (~ 20 kDa), this protein binds to the lipopolysaccharide (LPS) of Gram-negative bacteria and inhibits synthesis of outer membrane proteins. In a field trial, transgenic apple plants having an attacin E gene under control of the proteinase inhibitor II promoter showed reduction of symptoms of fire blight (*E. amylovora*) from 56% (non-transgenics) to 5% (transgenics) (Norelli *et al.*, 1999). The same gene

under control of a CaMV 35S promoter was introduced into pear plants (Reynold *et al.*, 1999). Transgenic shoots grown in tissue culture were evaluated for resistance against *E. amylovora* *in vitro*. Some of the lines were significantly more resistant than the original plant of the pear cultivar Passe Crassane, but not at the same level as the naturally resistant cultivar Old Home.

Analysis of all of these reports, both successful and unsuccessful, on the development of transgenic resistance against plant pathogenic bacteria through the production of cationic antimicrobial peptides shows that optimization of transgene expression and stability of the protein, allied to proper localization and timing, can result in significant disease reduction. In order to attain disease reduction, the observance of the following requisites seems to be necessary: (1) codons of the gene must not conflict with the codon usage of the host plant; (2) lytic peptides must be secreted in order to act upon the pathogen before cell damage occurs; (3) signal peptides for secretion must be correctly processed by the plant cell machinery; (4) lytic peptides must have reduced sensitivity to plant proteases; (5) promoters must confer strong gene expression preferentially in tissues that are colonized by the pathogen; and (6) promoters that are inducible by the presence of the pathogen are desirable since it avoids unnecessary expression and reduces the possibility of interference in plant development.

Xylem-specific Gene Expression

Because *X. fastidiosa* is a bacterium that inhabits xylem vessels, development of transgenic resistance against this pathogen requires constructs that cause the expressed protein to accumulate in this part of the plant. A few plant proteins are found in the xylem sap and the site of production of two of them is in the roots (Sakuta *et al.*, 1998). A better understanding of how these proteins are transported to the xylem vessels will allow the exploitation of this pathway to target antimicrobial peptides against *X. fastidiosa*. Nevertheless, this area of research is still incipient and the knowledge at the molecular level is limited to the cDNA sequences of only two xylem-

sap proteins found in cucumbers (Sakuta and Satoh, 1997). Xylem sap proteins that are associated with citrus blight have also been described by Derrick *et al* (1990). The serological detection of one of these in plant leaves, the 12 kDa protein, has been used as a diagnostic sign of the disease. Although the localization of this protein makes the use of its gene promoter appropriate for targeting peptides to the xylem sap, the fact that it is induced only in blight-affected trees precludes its use for the development of transgenic resistance against CVC.

A more realistic approach is the utilization of promoters from genes that are expressed preferentially in the xylem. Most of these genes encode enzymes involved in phenylpropanoid metabolism and synthesis of lignin precursors such as phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate:CoA ligase (C4L), cinnamyl alcohol dehydrogenase (CAD), caffeate 3-O-methyltransferase (COMT), and caffeoyl CoA 3-O-methyltransferase (CCoAOMT). Transgenic tobacco plants having a GUS gene under control of promoter sequences from a PAL gene of either *Arabidopsis*, common bean, pea, poplar or rice, showed expression of the reporter gene in young cells, below the stem apex, that were differentiating into xylem, as well as in cells of the xylem parenchyma in more developed parts of the plant (Bevan *et al.*, 1989; Gray-Mitsumune *et al.*, 1999; Kawamata *et al.*, 1997; Liang *et al.*, 1989; Ohl *et al.*, 1990; Zhu *et al.*, 1995). GUS expression could also be detected in epidermal cells and trichomes, root hairs and tip, and petals, where production of flavonoids requires PAL activity. Reporter gene induction by mechanical wounding and bacterial infection has also been demonstrated for PAL promoters (Giacomin and Szalay, 1996; Huang and McBeath, 1994).

The existence of multiple isoforms of PAL in plants confers flexibility in the expression of this enzyme, each gene being expressed in a particular spatial and temporal manner. This has been demonstrated using the promoters of PAL2 and PAL3 from French bean, fused to GUS and introduced into tobacco, potato, and *Arabidopsis* (Shufflebottom *et al.*, 1993). Among other tissue-specificity differences, a PAL2 promoter-GUS fusion showed the typical xylem-specific expression, while GUS expression under the PAL3 promoter was detected only in a single cell

layer surrounding the vascular system. The PAL3 promoter also showed an enhanced activity in response to wounding and to treatment with culture filtrates from *E. carotovora*.

Genetic Comparison Between Bacterial Strains

A CVC strain of *X. fastidiosa* was the first bacterial plant pathogen to have its genome completely sequenced. This accomplishment will soon be repeated for other plant pathogenic bacterial species. The availability of the complete sequence for one strain of a pathogen species helps in the understanding of the basic pathogenesis mechanisms of the other strains within the same taxonomic group. Nevertheless, very important aspects of the pathogen species such as host specificity, virulence, genetic diversity, and nutritional fastidiousness, among others, are not immediately elucidated by the analysis of the sequence of only one strain. Comparative genetic analysis of the sequenced strain with others closely related can address such issues and take advantage of the information already available.

Subtractive hybridization has been commonly used for these types of studies, but it is technically cumbersome and it may fail to recover DNA segments that contain a mixture of conserved and strain-specific stretches. Recently, a simpler procedure, called suppression subtractive hybridization (SSH), was developed for this type of study (Akopyants et al. 1998). This method involves ligation of adaptors to genomic DNA fragments digested with a frequent cutting restriction enzyme, hybridization of driver and tester samples, and PCR-amplification of tester-specific fragments. It was originally employed for subtraction of cDNA libraries from different human tissues (Diatchenko *et al.*, 1996) but has also been successful in detecting differences among strains of *Helicobacter pylori* (Akopyants *et al.*, 1998) and between *E. coli* and *Salmonella typhimurium* (Bogush *et al.*, 1999). Application of this procedure to find differences among strains of *X. fastidiosa* may result in data useful for the elucidation of their

phenotypic differences and the evolution of this pathogen species. The availability of the complete sequence of the CVC strain makes this information much more meaningful.

Objectives

1. To confirm the susceptibility of tobacco plants to infection by *X. fastidiosa*
2. To clone a PAL gene promoter from *Citrus sinensis*
3. To verify the tissue specific expression pattern conferred by the citrus PAL promoter in transgenic tobacco
4. To synthesize a cecropin gene optimized for expression in citrus plant cells
5. To express the cecropin gene under control of the citrus PAL promoter in transgenic tobacco plants and verify its effect on infection by *X. fastidiosa*
6. To employ suppression subtractive hybridization to find genetic differences between CVC and PD strains of *X. fastidiosa*

CHAPTER 2

INOCULATION OF TOBACCO PLANTS WITH *X. fastidiosa*

X. fastidiosa has a wide host range, being able to colonize both herbaceous and perennial plants. Periwinkle (*Catharanthus roseus*) can be infected by the PD and CVC strains of *X. fastidiosa* and could constitute a good model for host-pathogen interaction studies but the unavailability of a procedure to produce transgenic plants makes this species less attractive for this purpose. In his studies with this pathogen, Dr. Michael J. Davis (Tropical Research Center – Homestead, FL – University of Florida) inoculated tobacco plants with a PD strain of *X. fastidiosa* and observed that the bacterium could multiply in this host (personal communication). The cultivar of tobacco used in that experiment was not known. Since tobacco can be easily transformed via *Agrobacterium*, the utilization of this species as a model for the development of transgenic resistance against *X. fastidiosa* is a good alternative to the transformation of the original hosts, which are mainly perennials. Once the best strategy for transgenic resistance has been tested and selected in tobacco, it can then be applied to the relevant host species.

In order to confirm the observations made by Dr. M. J. Davis, tobacco plants of the species *Nicotiana tabacum* cv. Samsun and *Nicotiana benthamiana* were inoculated with a PD strain of *X. fastidiosa*. Plantlets about 10 cm high were inoculated with a suspension of *X. fastidiosa* in water (OD₆₀₀ ~0.5), by piercing the base of the stem several times with a hypodermic syringe needle through a small drop of the inoculum. Four plants of each tobacco species were inoculated.

After growing for two months in the greenhouse, the plants were assayed for the presence of *X. fastidiosa* by PCR using the primers RST31 and RST33 (Minsavage *et al.*, 1994). Although the

pathogen could be detected in all inoculated plants, symptoms were not clear and the inoculated plants could not be distinguished from the mock-inoculated controls.

One plant of each species was assayed for the presence of the bacterium in different segments of the stem by tissue immunoblot using a rabbit polyclonal antiserum raised against a CVC strain of *X. fastidiosa*. Segments of about 3-5 cm long were cut from the stem and printed on a nitrocellulose membrane. After drying, the membranes were blocked with 2% non-fat dry milk in TTBS (0.1 M Tris, 0.15 M NaCl, 0.1% Tween 20, pH 8.0) at 37°C for 60 min. Incubation with the polyclonal antiserum (1:30,000) and the alkaline phosphatase anti-rabbit conjugate (1:30,000) were performed at room temperature for 3 h each. Development was done with the substrates NBT and BCIP.

Figure 2-1 shows the results obtained for *N. tabacum* and *N. benthamiana*. *X. fastidiosa* could be detected throughout the stem and even in the upper part of the root in *N. tabacum*. Figure 2-2 shows a magnification of one of the prints, where the localization of the reaction to the xylem is clearly observable. These results show that the bacterium could multiply and systemically infect tobacco.

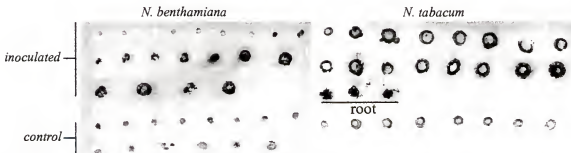


Figure 2-1. Tissue immunoprinting of tobacco plants inoculated with *X. fastidiosa*.

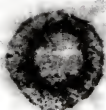


Figure 2-2. Detail of a tissue immunoprinting showing the reaction in the xylem.

CHAPTER 3 CLONING OF A PAL PROMOTER FROM SWEET ORANGE

Earlier generations of transgenic plants relied frequently on the use of strong, constitutive, and non-tissue specific promoters such as the CaMV 35S promoter. The use of appropriate gene promoters will be essential for the development of future generations of transgenics, in which they will confer gene expression in the target tissue, at the right time and intensity.

Whole genome sequencing projects are making available an enormous amount of information about genes, both in their coding and controlling regions, which can be used by molecular geneticists for the improvement of potentially any trait of a plant. In the near future, many plant species will have their genome sequenced. Most of this data will be generated by ESTs (expressed sequenced tags) projects, for the obvious reasons that those are less expensive and faster than whole genome sequencing, and because the most valuable information resides in the coding region of the genes. With this information, it is possible to assess spatial and temporal patterns of gene expression by Northern blots (few genes at once) or by microarrays (hundreds or thousands of genes at once). From this analysis, genes that show a pattern of expression that is desirable to reproduce for a particular transgene can be selected to have their promoters cloned.

PAL promoters from several plant species have been isolated and shown to control gene expression in tissues where phenylpropanoids are actively produced, such as the xylem parenchyma and flower petals (Bevan *et al.*, 1989; Gray-Mitsumune *et al.*, 1999; Kawamata *et al.*, 1997; Liang *et al.*, 1989). The expression in xylem tissues is a feature that makes this promoter appropriate for the development of transgenic resistance against *X. fastidiosa*.

Although, in most cases, the use of a promoter cloned from one plant species confers the same tissue specificity when introduced into a different species, it has been reported that a PAL promoter from poplar conferred a β -glucuronidase (GUS) expression pattern in transgenic tobacco that differed from that in the original species (Gray-Mitsumune *et al.*, 1999). Therefore, the use of endogenous promoters is preferable to ensure transgene expression in the desired tissue.

Usually, to obtain genomic sequences that flank the coding region of a particular gene, one uses the latter as a probe to screen a genomic library. Once a cosmid, bacterial artificial chromosome (BAC), or yeast artificial chromosome (YAC) clone is identified, sub-clonings are necessary to get fragments small enough to be sequenced. This can be a long and tedious process, especially if a genomic library is not yet available. An alternative to this is to employ Inverse PCR (IPCR) to obtain unknown DNA sequences which flank a region whose sequence is already known (Ochman *et al.*, 1988; Triglia *et al.*, 1988). In this procedure, primers are designed facing outward from a core region and restriction enzymes that cut just outside this region are used separately to digest genomic DNA. After digestion, the DNA is diluted and circularized by addition of DNA ligase. Dilution conditions that favor the formation of monomeric circles are important. The circularized DNA is used as template in a PCR reaction employing the inverse primers. The amplification product will include segments both 5' and 3' to the core region. By selecting a restriction enzyme that cuts next to the 5' or 3' end of the core region, it is possible to obtain downstream or upstream sequences, respectively. The size of the amplified flanking segment will depend on how far from the core region the next site for the selected restriction enzyme is located, and also on the efficiency of the PCR method employed. Figure 3-1 describes schematically the IPCR procedure.

In order to clone the promoter of a PAL gene from sweet orange, a search for this gene was performed in the GenBank (<http://www.ncbi.nlm.nih.gov/Entrez/>). A cDNA sequence from

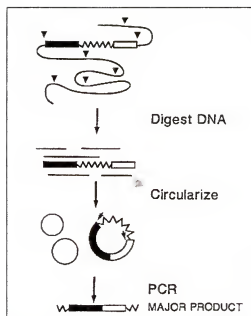


Figure 3-1. Schematic representation of inverse PCR. The core region is represented by a jagged line and flanking regions by filled and open boxes. Triangles represent sites recognized by the restriction enzyme. Inverse primers are represented by small arrows pointing outward from the core region (from Ochman *et al.*, 1988).

Citrus limon (GenBank accession number U43338) was found. Protein sequence alignment revealed the highly conserved nature of this gene among many plants (Figure 3-2). Having this information, it was reasonable to assume that using primers designed based on the *C. limon* PAL sequence, the homologous gene from *C. sinensis* could be amplified by PCR.

A BLAST (Basic Local Alignment Search Tool) search using the lemon PAL cDNA sequence showed that an intron was always present in genomic PAL genes, and always interrupted the coding region at approximately the same position (Figure 3-3).

Three primers were designed to amplify fragments of a PAL gene from sweet orange. Figure 3-4 shows their annealing position in the sequence of the lemon PAL cDNA. PCR using primers PAL1 (5'-AGCGGACTCATTGAAAGG-3') and PAL3 (5'-TACCTAATGAGCTCCTTTTG-3') should amplify a 309 bp fragment of the first exon. Although the primer PAL4 (5'-CCAAAAAT TCCAGAATTCAA-3') is adjacent to PAL3, based on the cDNA sequence, it was designed to

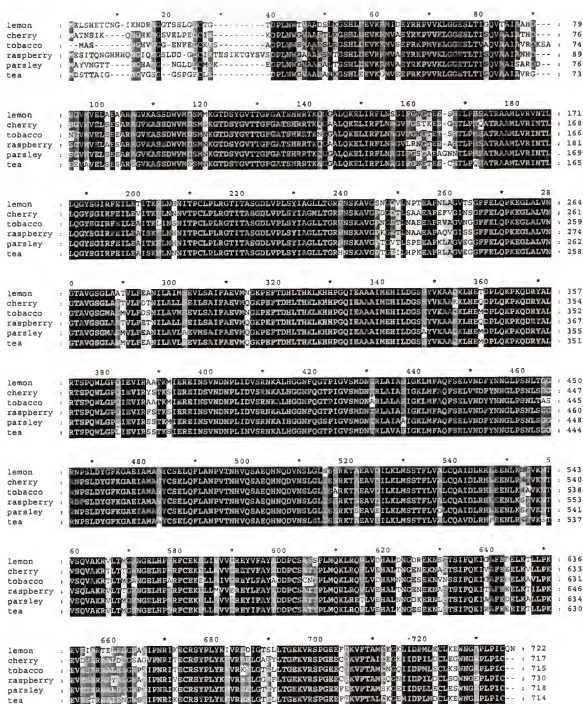


Figure 3-2. Alignment of PAL protein sequences from different plant species showing the highly conserved nature of these proteins. Lemon = *Citrus limon*, cherry = *Prunus avium*, tobacco = *Nicotiana tabacum*, raspberry = *Rubus idaeus*, parsley = *Petroselinum crispum*, tea = *Camellia sinensis*

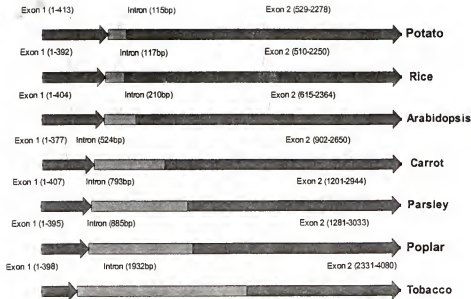


Figure 3-3. Presence of introns in PAL genomic sequences from different plant species. Potato = *Solanum tuberosum*, rice = *Oryza sativa*, Arabidopsis = *Arabidopsis thaliana*, carrot = *Daucus carota*, parsley = *Petroselinum crispum*, Poplar = *Populus kitakamiensis*, Tobacco = *Nicotiana tabacum*.

anneal to the 5' end of the second exon, therefore, together with primer PAL1, it should amplify part of the first exon plus the whole predicted intron.

Genomic DNA was extracted from leaves of Meyer lemon (*C. limon*) and Madam Vinous sweet orange (*C. sinensis*) using a CTAB procedure (Ausubel *et al.*, 1997). Primers PAL1 + PAL3 and PAL1 + PAL4 were used to PCR amplify fragments of the PAL gene from both citrus species. Approximately 100 ng of genomic DNA was used as template along with 200 nM of each primer, 200 μ M of dNTPs, 2.5 U of Taq polymerase (Promega), in a buffer containing 2.5 mM of $MgCl_2$ and final volume of 50 μ l. The PCR program consisted of an initial denaturation at 94°C for 5 min, followed by 40 cycles of 94°C/30s, 54°C/30s, 72°C/30s (PAL1 + PAL3) or 72°C/3 min (PAL1 + PAL4), and final extension at 72°C/10 min. The PCR products were analyzed in a 1.5% agarose gel in TAE buffer. The expected product of 309 bp for the primers PAL1 + PAL3 was detected for both lemon and sweet orange (Figure 3-5). With primers PAL1 +

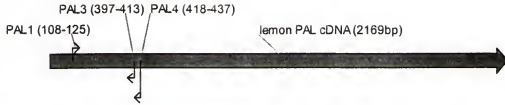


Figure 3-4. Position of primers PAL1, 3, and 4 in the lemon PAL cDNA sequence

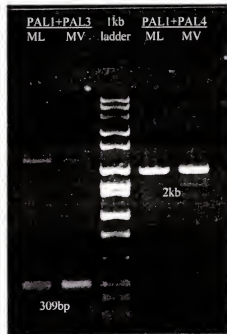


Figure 3-5. PCR products obtained with primers PAL1+PAL3 (309bp) and PAL1+PAL4 (~2Kb) from genomic DNA from Meyer lemon (ML) and Madam Vinous sweet orange (MV). 1Kb ladder (Sigma) was used as size standards

PAL4, a product of around 2 Kbp was amplified which indicated that an intron of approximately 1700 bp occurred between the first and second exons, both in lemon and sweet orange.

The product obtained from Madam Vinous sweet orange using primers PAL1 + PAL4 was cloned in pGEM-T (Promega) and designated as MV1-4. This clone was partially sequenced, starting from both 5' and 3' ends. All sequencing reactions in this study were performed at the Interdisciplinary Center for Biological Research (ICBR) Sequencing Core Lab using the

fluorescent dye-terminator procedure. Alignment of the 5' end of MV1-4 with the lemon PAL cDNA sequence available in the GenBank shows the strong homology between the two genes and the presence of an intron starting at the predicted site (Figure 3-6). The inverse primers PAL7 (5'-TAGGCAAAGT GAAGTAGTTCCA-3') and PAL8 (5'-ATGGACAGCATGATGAAAGGG-3') were designed based on the sweet orange sequence. Figure 3-7 represents the whole MV1-4 clone and shows the position of the primers and of the restriction sites for the enzymes *Apa*I, *Dra*I, *Hind*III, *Sac*I, and *Ssp*I that were selected to perform the I-PCR.

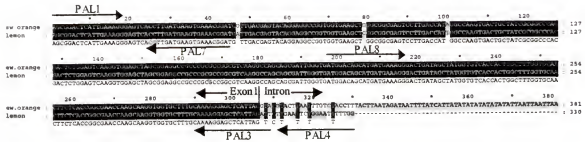


Figure 3-6. Comparison of the 5' end of the sweet orange genomic clone MV1-4 with the correspondent region of PAL lemon cDNA.

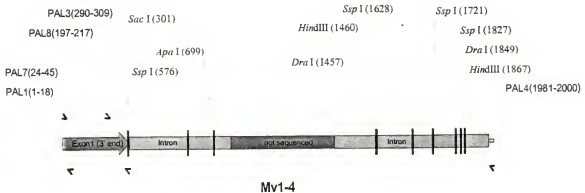


Figure 3-7. Schematic representation of clone MV1-4, showing the positions of primers and restriction sites chosen for the inverse PCR procedure.

For the inverse PCR procedure, 15 µg aliquots of Madam Vinous sweet orange genomic DNA were digested separately with 10U of each of the selected restriction enzymes for 8h at 37°C. One microgram from each digest was diluted in 100 µl of ligase buffer and 1 µl of T4 DNA ligase (3 U) was added to each sample. Circularization reactions were incubated overnight at 14°C. Five microliters of the circularized DNA (50 ng) was submitted to PCR with primers PAL7 and PAL8 (500 nM each), 200 µM dNTPs, 1 µl of Elongase enzyme mix (Gibco BRL), MgCl₂ at 1.5 mM, in a 50 µl final volume. The thermocycler program consisted of an initial denaturation step of 94°C/3 min followed by 35 cycles of 94°C/30s, 58°C/60s, 68°C/5min. PCR products were visualized in a 1% agarose gel in TAE buffer (Figure 3-8). A strong band of about 3 Kb was detected for the sample digested with SspI and faint bands were obtained from the DraI and HindIII digests.

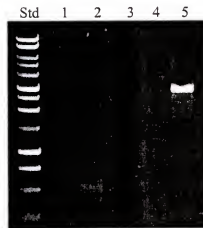


Figure 3-8. Inverse PCR products from Madam Vinous sweet orange genomic DNA digested with restriction enzymes (1)Apal, (2)DraI, (3)SacI, (4)HindIII, and (5)SspI. Std =1 Kb ladder (Promega).

The product amplified from the SspI digested and circularized DNA was cut from the agarose gel and extracted using a QIAquick gel extraction kit (Qiagen). The purified product was ligated to pGEM-T, generating the clone Ssp4, and its end containing the PAL7 primer was

sequenced to check for the presence of sequences upstream from the PAL gene. The whole *Ssp4* clone was sequenced, by producing subclones *Ssp4/9-10* (amplified by PCR using primers PAL9, 5'-TCTATACCATTATGCACG-3' and PAL10 5'-ACAATTCAACCATCT GAT-3') and *Ssp4-delNde* (obtained by deleting a *NdeI* fragment from *Ssp4/9-10*) (Figure 3-9).

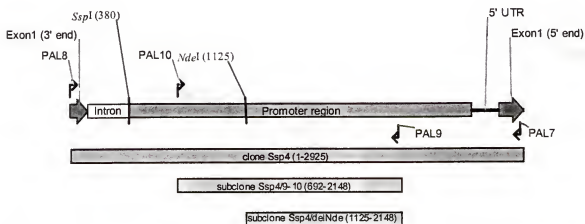


Figure 3-9. Clone *Ssp4* and subclones *Ssp4/9-10* and *Ssp4/delNde*, were produced for sequencing the whole inverse PCR product.

Alignment of the sweet orange sequence upstream from primer PAL7 with the cDNA sequence of lemon shows a strong homology between them, with the putative translational start site located at the same position (Figure 3-10). Analysis of the sweet orange sequence using promoter prediction software (http://www.fruitfly.org/seq_tools/promoter.html/) indicated a putative transcriptional start site that differs from the lemon cDNA start by one base. Consensus CAAT and TATA sequences upstream from the putative transcriptional start site can be recognized.

Figure 3-11 shows the assembled sequence of clone Ssp4. Upstream from primer PAL7, beyond the translational and transcriptional start sites, there are 2220 nucleotides of the promoter region until reaching the SspI restriction site. Downstream from primer PAL8 were sequences corresponding to the 3' end of exon1 of the PAL gene followed by 265 nucleotides of the intron, until reaching the SspI restriction site.

The region of the PvPAL2 corresponding to Box P has been demonstrated to be necessary for the xylem specificity of this promoter (Leyva *et al.*, 1992). Deletion of this segment lead to

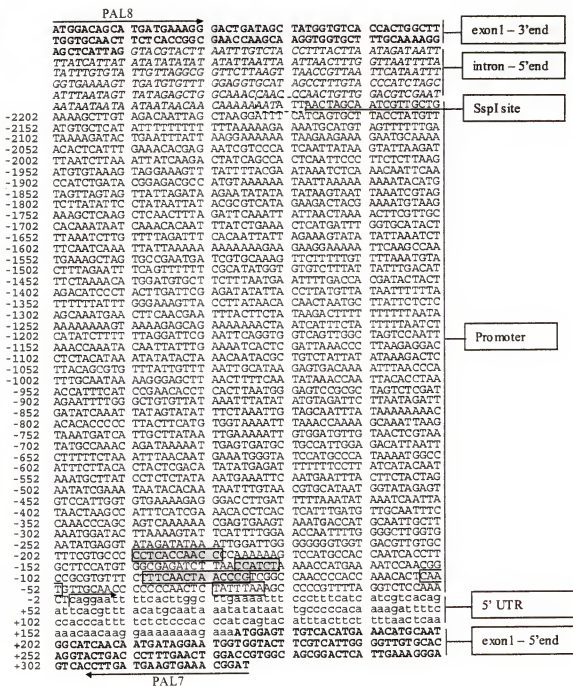


Figure 3-11. Complete sequence of clone Ssp4 showing the 5' and 3' ends of exon1 (**BOLD**), 5' end of the intron (*ITALICS*), promoter sequences (**CAPITAL**), 5' UTR (lowercase), CAAT and TATA boxes (open boxes), SspI site (dotted box), putative transcription start site (broken arrow), consensus cis-elements of PAL promoters (shaded boxes), and binding sites for primers PAL7 and PAL8 (arrows).

loss of GUS expression in xylem and allowed the manifestation of upstream cryptic cis-element(s) that caused GUS expression in the phloem. The Box P, also called element AC-III, is the target for the Myb-like transcriptional activator from snapdragon (Myb305) (Sablowski *et al.*, 1995). Myb-like factors are known to control the expression of genes involved in phenylpropanoid metabolism in plants (Martin and Paz-Ares, 1997).

Promoter	Box P	Position*	Box A	Position*	Box L	Position*
PcPAL	CTCCAACAAACCCC	-175	CCGTCC	-160	TCTCACCTACC	-107
PcPAL	CTCCAACAAACCCC	-175	CCGTCC	-160	TCTCACCTACC	-107
PcPAL	TTCCAACAAACCCC	-178	CCGTCC	-163	TCCCACCTACC	-98
PcPAL	CTCCAACCATCCAA	-183	CCGTTC	-136	TCTCACCAACC	-268
Pc4CL1	CTTTACCAACCCCC	-52	CCGTCC	-228	TCTCACCAACC	-128
Pc4CL2	CTTTACCAACCCCC	-52	CCGTCC	-228	TCTCACCAACC	-128
AtPAL1	TCTCAACCAACTCC	-135	CCGTGT	-260	GCTTACCTACC	-51
AtPAL2	TCTCACCCACCCCT	-132	?		CCTTACCTAAC	-55
LePAL5	TTCTTACAACCCCC	?	?		CTTACCTACC	?
PsPAL1	ATTCAACAAACCCAC	-136	CCATCC	-265	CCTCACCTACC	-92
PsPAL2	CTCAACCAAACCCAC	-204	CGCTCC	-227	CTTACCTACC	-151
PvPAL2	TCTCCACCAACCCCC	-123	ACGTCC	-274	ACCCACCTACC	-76
PvPAL3	CAACCACCTACCCC	-77	?		ACTCACCAACC	-53
StPAL1	TTCCAACAACCCACC	-165	CCCTCT	-136	TCCCATCTCCA	-83
StPAL2	CTTCAACAACCCACC	-169	CCCTCC	-140	TCTCATCTACC	-72
St4CL1	CTTTACCTACCCAC	-57	CTGTCC	-476	TCTCACCAACC	-170
St4CL2	CTTTACCTACCCAC	-58	CTGTCC	-476	TCTCACCAACC	-168
Consensus	C TCAA A A CC T T C A A C C T CTCC C C AA		CCGTCC		T TC T C C ACC ACC C CT A	
CsPAL	TTTCAACTAACCCG	-78	CCATCT	-124	CCTCACCAACC	-182

Table 3-1. Putative cis-acting elements and their positions in various PAL and C4L gene promoters (from Logemann *et al.*, 1995; CsPAL from this work). At = *Arabidopsis thaliana*, Le = *Lycopersicon esculentum*, Pc = *Petroselinum crispum*, Ps = *Pisum sativum*, Pv = *Phaseolus vulgaris*, St = *Solanum tuberosum*, Cs = *Citrus sinensis*. Mismatches on CsPAL are represented by small cap letters. *Position relative to transcription start site.

CHAPTER 4

TRANSFORMATION OF TOBACCO PLANTS WITH THE GUS GENE UNDER CONTROL OF THE CITRUS PAL PROMOTER

In order to demonstrate that the PAL promoter cloned from sweet orange confers xylem-specific gene expression, a construct containing the GUS gene under control of this promoter was made for the transformation of tobacco plants via *Agrobacterium*.

A 2073 bp fragment from the citrus PAL promoter was PCR-amplified from the Ssp4 clone using primers PAL10 (previously described) and PAL12 (5'-AAACCATGGTTTCCTTGTTGTTTGTAG-3'). The PAL12 primer is complementary to nucleotides +146 to +164 in the 5' UTR of the PAL promoter, and introduces a NcoI restriction site near its 5' end. The PCR program consisted of an initial denaturation step of 94°C/3min followed by 40 cycles of 94°C/30s, 58°C/30s, 72°C/3min, and final elongation for 10min. The amplified product was gel purified and ligated to a pGEM-T vector. The resulting clone was denominated CsPAL10-12 (Figure 4-1).

A 1346 bp EcoRI-NcoI fragment of the PAL promoter was digested from the CsPAL10-12 clone and ligated to a pCAMBIA 1201 binary vector (Figure 4-2) digested with the same enzymes. This cloning resulted in the substitution of the citrus PAL promoter for the CaMV 35S promoter of the GUS gene in the pCAMBIA vector. The clone was named CsPP-GUS/1201 (*Citrus sinensis* PAL Promoter) (Figure 4-3).

The pCAMBIA 1201 vector has a hygromycin resistance gene for the selection of transgenic plant shoots. Since, for practical reasons, the use of kanamycin resistance was more convenient in our lab, the EcoRI-BstEII fragment containing the PAL promoter and the GUS gene was digested from the CsPP-GUS/1201 clone and ligated to the pCAMBIA 2201 vector digested with the same enzymes. The citrus PAL promoter could not be cloned directly in this vector because of the

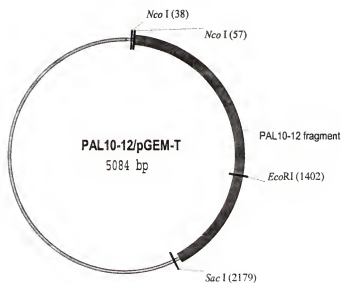


Figure 4-1. Clone PAL10-12/pGEM-T containing a 2073 bp PCR product amplified from the citrus PAL promoter.

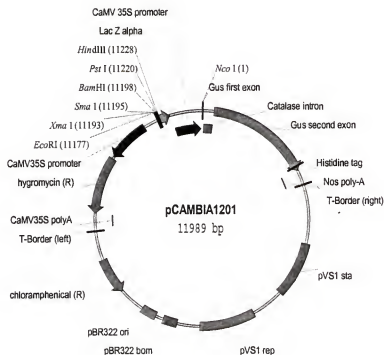


Figure 4-2. Binary vector pCAMBIA 1201.

presence of a second NcoI site in the kanamycin resistance gene. The resulting clone was designated CsPP-GUS/2201 (Figure 4-4). Correctness of the construct was checked by digestion with different restriction enzymes and by subcloning and sequencing of a 812 bp MunI fragment that encompasses the 3' end of the CsPP and the 5' end of the GUS gene (Figure 4-4).

In order to compare the sweet orange PAL gene promoter activity with another one that has already been shown to confer xylem-specific gene expression, an *Arabidopsis thaliana* PAL gene promoter was cloned by PCR. Primers AtPAL1 (5'-CACGAAAAATGCAAAAGACTG-3') and AtPAL2 (5'-CCATGGCAATAGAAGATTAAGAGGAGATTAC-3') were designed to amplify a 1961 bp fragment of a PAL gene promoter from *A. thaliana*, which corresponded to the same fragment used in the study by Ohl *et al.* (1990). The primers were based on the genomic sequence available for the PAL1 gene, which is found in section 202 (GenBank accession number AC006260) of chromosome II, completely sequenced by the *Arabidopsis* Genome Initiative. Primer AtPAL2 introduces a NcoI restriction site and 14 bases downstream from primer AtPAL1 there is a restriction site for EcoRI, both of which allow cloning of the promoter in the pCAMBIA 1201 vector. Genomic DNA was extracted from *A. thaliana* ecotype Columbia leaves using a CTAB-based method and the PAL gene promoter was amplified by PCR using the following program: initial denaturation at 94°C/3min, 35 cycles of 94°C/20s – 54°C/30s – 68°C/2 min, and final extension at 68°C/5 min. The PCR product was gel purified, cloned in pGEM-T, and partially sequenced from both ends to compare with the published sequences. A 1922 bp fragment from the *A. thaliana* PAL promoter was removed from the pGEM-T vector by digestion with NcoI and EcoRI, and cloned in the pCAMBIA 1201 digested with the same enzymes, giving rise to the clone AtPP-GUS/1201. The EcoRI-BstEII fragment containing the AtPP-GUS fusion was transferred to the pCAMBIA 2201 vector, originating the AtPP-GUS/2201 clone (Figure 4-5). Correctness of the construct was checked by digestion with different restriction enzymes and by subcloning and sequencing of a 982 bp EcoRV-MunI fragment that encompasses the 3' end of the AtPP and the 5' end of the GUS gene (Figure 4-5).

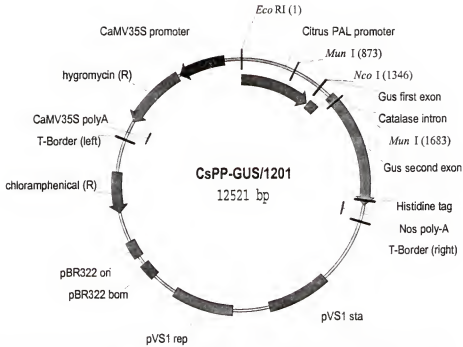


Figure 4-3. Citrus PAL promoter fused to GUS in the binary vector pCambia 1201.

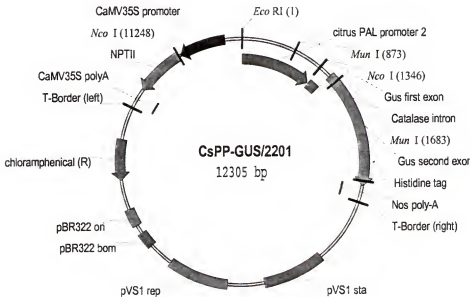


Figure 4-4. Citrus PAL promoter fused to GUS in the binary vector pCambia 2201.

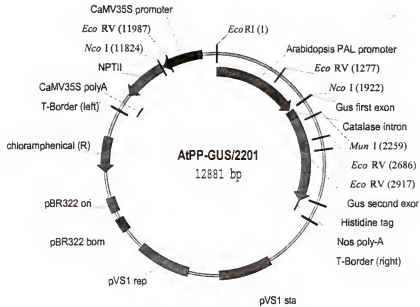


Figure 4-5. *A. thaliana* PAL promoter fused to GUS in the binary vector pCambia 2201.

The CsPP-GUS/2201 and ATPP-GUS/2201 vectors were transferred from *E. coli* to *A. tumefaciens* GV2260 by triparental mating, using *E. coli* pRK2013 as helper. Transconjugants were selected in LB plates containing rifampicin (75 µg/ml) and chloramphenicol (25 µg/ml). Confirmation of the presence of the vector in transconjugant colonies was done by PCR using primers PAL10 + PAL12 (for CsPP) and AtPAL1 + AtPAL2 (for AtPP).

Tobacco plants (*N. tabacum* cv. Samsun) were transformed by co-cultivation of leaf disks with *A. tumefaciens* GV2260 containing the binary vector CsPP-GUS/2201 or ATPP-GUS/2201. Tobacco seeds were superficially disinfected by treatment with 100% ethanol for a few seconds, followed by 0.5% Na hypochlorite/1% SDS for 5 min, and washing with sterile water four times. Plantlets were aseptically grown from seeds germinated on R1/2N medium (2.15 g Murashige Skoog salt mixture – Gibco-BRL, 50 mg myo-inositol, 18.65 mg Na₂EDTA, 13.9 mg FeSO₄·7H₂O, 10 g sucrose, 6 g agar, for 1 liter of medium, pH adjusted to 5.8). Overnight liquid cultures of *A. tumefaciens* were centrifuged and resuspended in 0.5x MS salts solution (2.15 g/l), to an OD₆₀₀ of approximately 0.5. Leaf disks of 0.8 cm in diameter were cut using a cork borer

and floated on the *A. tumefaciens* suspension for 10 min. After removing excess bacterial suspension by blotting on filter paper, the leaf disks were placed on solid DBI medium (4.3 g MS salt mixture, 100 mg myo-inositol, 4 mg thiamine, 30 g sucrose, 8 g agar, for 1 l of medium, pH adjusted to 5.6), incubated in the dark at room temperature (22-24°C) for 48 h and transferred to DBI medium supplemented with the plant hormones IAA (1 mg/ml) and kinetin (2 mg/ml), and the antibiotics kanamycin (50 mg/ml) and mefoxin (200 mg/ml). The plates were kept in a growth chamber with cycles of 16h light/8h dark, at 28°C. Two to three weeks later, and thereafter, shoots that grew from the borders of the leaf disks were transferred to rooting medium R1/2N supplemented with kanamycin (50 mg/ml) and mefoxin (200 mg/ml) and kept in a growth chamber under the same conditions as before. Roots usually formed one or two weeks later, and rooted shoots were transferred to 'baby food' jars containing autoclaved potting mix. After growing in the growth chamber for about 2-3 weeks, the plantlets were transferred to 8" pots and kept in the greenhouse.

Initial screening of the transgenic plantlets was done by GUS assay. Small pieces cut from the tip of the leaves and transverse sections of petioles were incubated in X-gluc solution (1 mg/ml in 0.1 M phosphate buffer pH 7.0, 10 mM Na₂EDTA) overnight at 37°C. Plants that were negative for GUS were discarded, and positive plants were kept for further GUS assays of more mature tissues.

Leaf petioles from transgenic plants grown for two months in the greenhouse (30-40 cm tall) were hand-sectioned transversally using a razor blade and incubated with 0.5% formaldehyde in 0.1M sodium phosphate buffer (pH 7.0) for 30 min at room temperature. The sections were washed 4 x 10 min with phosphate buffer and stained with X-gluc solution overnight at 37°C.

Petioles from CsPP-GUS transgenic plants showed GUS staining throughout the transverse section, but a much more intense color could be seen in a layer of young xylem cells (Figure 4-6). Identical patterns were observed in five independent transgenic lines.

GUS staining of leaf petioles from AtPP-GUS transgenic tobacco showed a pattern that has been described previously for the other PAL promoters (Bevan *et al.*, 1989; Liang *et al.*, 1989). Cells surrounding xylem vessels, corresponding mainly to the xylem parenchyma, were intensely stained (Figure 4-7). The same pattern was observed in the two transgenic lines analyzed.

The GUS reaction was much faster for the CsPP plants, with blue color being observable as soon as 1 h of incubation at 37°C. The AtPP plants required at least 6 h of incubation. Also, during these assays, sections from the CsPP plants conferred a blue color to the X-gluc solution that was not observed for sections from the AtPP plants, even after several hours of incubation. This probably results from the lower tissue-specificity obtained with the sweet orange promoter, allowing GUS expression in cortical cells. These large cells were ruptured when the sections were made, and their contents were released into the staining solution.



Figure 4-6. GUS staining of transverse sections of leaf petioles from CsPP-GUS transgenic tobacco.



Figure 4-7. GUS staining of transverse sections of leaf petioles from AtPP-GUS transgenic tobacco.

These results indicate that the PAL promoter cloned from sweet orange preferentially, but not exclusively, confers gene expression in xylem tissues. Weaker GUS staining was also detected throughout the petiole section. The promoter cloned from *Arabidopsis* conferred GUS expression exclusively in the xylem parenchyma, in accordance with what has already been observed by Ohl *et al.* (1990). It is possible that these differences are a consequence of using a smaller fragment, 1346 bp, from the sweet orange promoter in the CsPP-GUS construct, when compared to the 1922 bp used from the *Arabidopsis* promoter in the AtPP-GUS construct. It has been demonstrated that the PAL promoter from bean (Leyva *et al.*, 1992) and from *Arabidopsis* (Ohl *et al.*, 1990) are composed of a mosaic of positive and negative cis-elements. Although, for most of the PAL promoters analyzed, the elements that confer xylem-specificity are concentrated within the first 1 kb upstream from the transcriptional start site, the sweet orange PAL promoter may have a different distribution of elements and binding sites for repressors could be located upstream from the 1346 bp used in this study.

CHAPTER 5

SYNTHESIS OF A CECROPIN GENE AND EXPRESSION IN TRANSGENIC TOBACCO UNDER CONTROL OF THE CITRUS PAL PROMOTER

The effect of synthetic cecropin A (Sigma) on *X. fastidiosa* was verified using an “in vitro” assay. Two hundred microliter aliquots of a five day old liquid culture of a PD strain of *X. fastidiosa* (OD₆₀₀ ~0.5) were incubated overnight at 28°C with cecropin A at final concentrations of 0.25 µM, 0.5 µM, 1 µM, 2 µM and 4 µM. *X. fastidiosa* suspension culture without cecropin was used as control. After this incubation, serial dilutions were made from each of the tubes and plated on solid PW-H medium (Davis *et al.*, 1981). Plates were incubated at 28°C and colonies were counted 10-14 days later. No colonies grew on plates corresponding to the treatment with cecropin A at 4 µM while, at 0.25 µM, there was no difference in colony numbers in comparison with the control. Treatments with concentrations of 0.5 µM, 1.0 µM, and 2.0 µM, caused reduction in colony numbers of approximately 10, 100, and 1000 fold, respectively. These results indicate that *X. fastidiosa* is sensitive to cecropin A at a minimum inhibitory concentration (MIC) of 0.5 µM, which is in agreement with what has been reported for other plant pathogenic Gram-negative bacteria (Owens and Heutte, 1997).

A cecropin gene was synthesized by joining and extending oligonucleotides containing the proper coding sequence, using a PCR procedure. There are several advantages in doing so: (1) codons can be chosen according to the codon preference table of the species to which the gene will be introduced, avoiding incompatibilities between the translational machinery of the donor species and the recipient one; (2) restriction sites are introduced at will to facilitate cloning steps; and (3) modifications in the peptide sequence that confer higher activity or stability can be introduced.

Other methods for cloning anti-microbial peptides have been reported. For example, synthesis of genes for different antibacterial peptides and expression as fusion proteins with protein A in *E. coli* has been reported by Martemyanov *et al.* (1996). Each gene was constructed by annealing sets of complementary oligonucleotides and joining them using DNA ligase to compose the entire sequence. An alternative method to synthesize genes that is more conservative in oligonucleotides usage was described by Di Donato *et al.* (1993). Instead of using oligonucleotides that are complementary in their entire length, they employed partially overlapping ones and extended them using a PCR procedure. Initially, a “core template” corresponding to the middle of the gene was produced and in subsequent steps, it was extended at both extremities using new oligonucleotides that partially overlap to these ends. Using this method and 10 oligonucleotides in the range of 42 to 62 bases long, a human liver ribonuclease gene, 573 bp long, was synthesized by these authors. Figure 5-1 represents this procedure schematically.

For the present study, a modified cecropin (MB39) that is more resistant to proteases from different plant species was selected for synthesis (Owens and Heutte, 1997). Four oligonucleotides, between 62 to 68 bases long, were designed having the codons corresponding to the MB39 cecropin amino acids preceded by a signal peptide for export. The secretory sequence from the barley amylase gene was used because there is evidence that it can be processed correctly and can target cecropin peptides to the intercellular spaces of plant tissues (Huang *et al.*, 1997). Codons were chosen according to the codon usage tables for *Citrus sinensis* and citrus tristeza virus (CTV) (<http://www.kazusa.or.jp/codon/>). Since the number of sequenced genes for sweet orange is not large, we have also taken into account the codon usage of CTV, assuming that this virus is efficient in using the host cell translational machinery and its codon usage is well adapted to this plant. When a particular codon conferred a highly stable secondary structure to the oligonucleotide, as indicated by primer analysis software (<http://www.idtdna.com/html/analysis/Calculator.html>), it was switched to the second most used one. Restriction sites for the

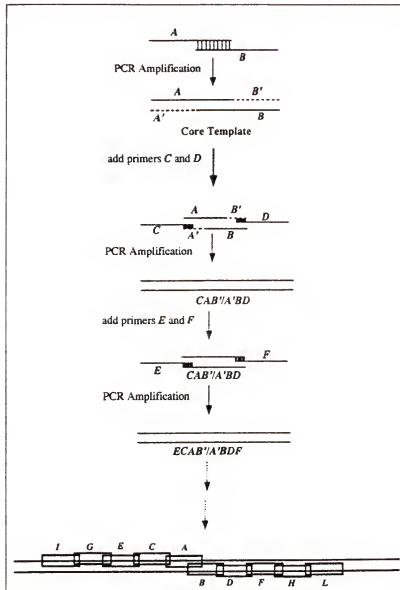


Figure 5-1. Schematic representation of the procedure used for the synthesis of a modified cecropin gene (from Di Donato *et al.*, 1993).

enzymes *Xba*I and *Nco*I, at the 5' end, and *Xho*I, at the 3' end, were introduced for cloning purposes. Sequences in accordance with the Kozak consensus were used at the translational start site. Figure 5-2 represents schematically the 211 nucleotides of the whole gene and the oligonucleotides that were used to construct it. Oligos CEC2 and CEC3 are partially complementary at their 3' end, oligo CEC1 partially overlaps the 5' end of CEC2 and oligo CEC4 partially overlaps the 5' end of CEC2.

The oligonucleotides were purchased from Integrated DNA Technologies, Coralville-IA, without any special purification (desalted by passage through a Sephadex G25 column only). The core part of the gene was assembled using oligos CEC2 and CEC3. Two hundred nanomolar of each oligo were annealed, extended, and amplified through PCR using 200 μ M of dNTPs, 4.5U of KlenTaq polymerase (Sigma), and buffer supplied with the enzyme with 1.5 mM $MgCl_2$ in a total volume of 50 μ l. The tube containing all the components, except the KlenTaq polymerase, was heated to 94°C for 3 min and slowly cooled down, over a 22 min period, to the annealing temperature of 54°C. The enzyme was added and the oligos were extended at 68°C for 1 min. Subsequently, 24 cycles of 94°C/30s and 68°C/60s were applied, followed by a final extension of 3 min at 68°C. Two microliters of this reaction was submitted to a second PCR using oligos CEC1 and CEC4, using the same conditions. Five microliters of each PCR reaction was analyzed in a 3% NuSieve agarose (FMC) gel in TAE buffer. Figure 5-3 shows the products obtained after each reaction, with the predicted sizes of 107 bp and 211 bp.

The PCR product obtained after the second reaction was gel purified using a QIAquick gel purification kit (QIAGEN) and cloned in pGEM-T (Promega). Five clones were submitted to sequencing, and all of them showed one or two errors (deletion or insertion of bases), at different points of the gene. These errors were attributed to misincorporations during the synthesis of the oligos, which are common in long molecules, and that could have probably been eliminated with a more rigorous purification procedure (HPLC or PAGE). One clone that showed one base deletion was corrected through PCR employing primers that introduced the missing base. The corrected clone was verified by sequencing and named CEC/pGEM-T.

In order to express the cecropin gene in tobacco, a construct was made placing the gene between the citrus PAL promoter and the CaMV 35S terminator. Figure 5-4 represents schematically the cloning strategy. Initially, the cecropin gene was removed from the CEC/pGEM-T plasmid by digestion with XbaI and XhoI and ligated to pUC118/35S P-T (kindly provided by Dr. Vicente J. Febres) digested with the same enzymes. The resulting plasmid,

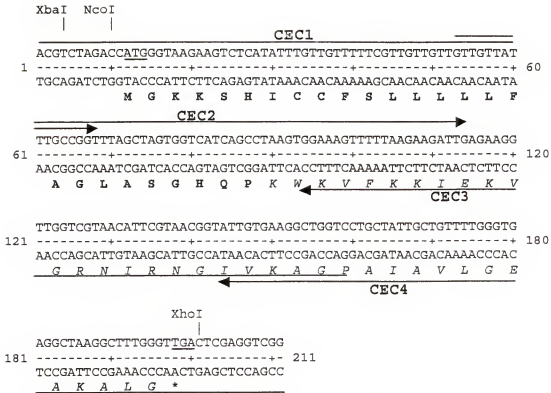


Figure 5-2. Sequence of the modified cecropin MB39 and the oligonucleotides (arrows) that were employed to construct it. Amino acids in bold represent the signal peptide and, in italics, the mature cecropin. Start and stop codons are underlined.

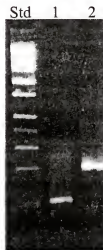


Figure 5-3. Synthesis of the cecropin MB39 gene through PCR. Std = 100 bp ladder, 1 = product of the first PCR reaction, 2 = product of the second PCR reaction

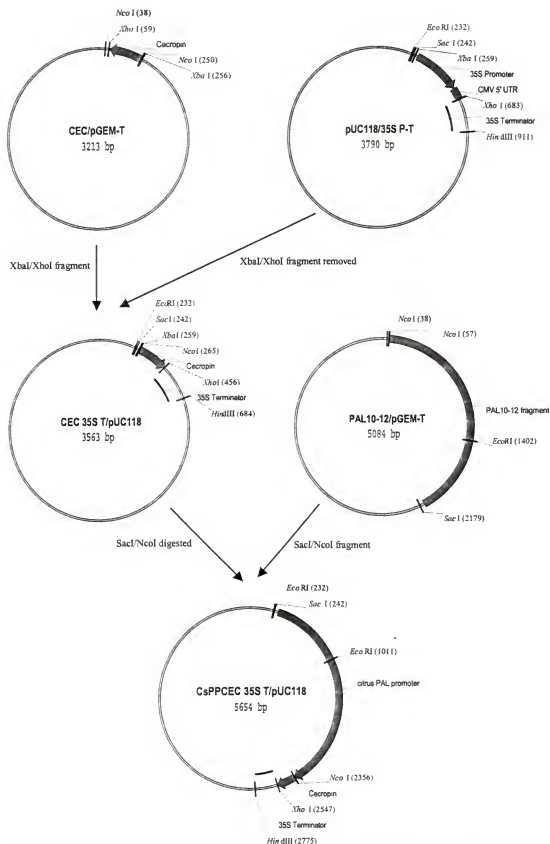


Figure 5-4. Cloning strategy for the construction of a plant expression cassette containing the cecropin gene under control of the citrus PAL promoter

named CEC 35S T/pUC118, had the cecropin gene followed by the CaMV 35S terminator. In the next step, the CsPP was removed from the clone CsPAL10-12/pGEM-T (previously described) by digestion with *SacI* (site present in the vector) and *NcoI* (introduced by the primer PAL12) and ligated to CEC 35S T/pUC118 digested with the same enzymes. The resulting construct was named CsPPCEC 35S T/pUC118.

The *SacI*/*HindIII* fragment was removed from CsPPCEC 35S T/pUC118 and ligated to the binary vector pCambia 2201 digested with the same enzymes, generating the clone CsPPCEC/2201 (Figure 5-5). The correctness of the construct was verified by digestion with a combination of restriction enzymes and by sequencing of an *EcoRV*/*HindIII* fragment containing the 3' end of the CsPP, the cecropin gene, and the CaMV 35S terminator. This construct was

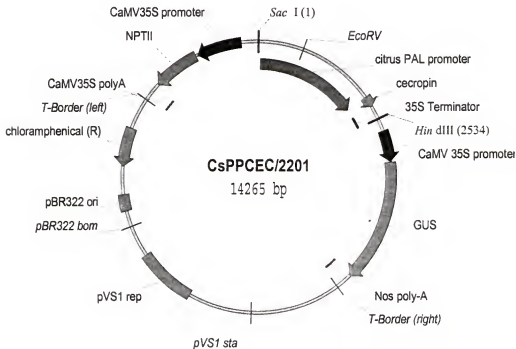


Figure 5-5. Cecropin gene under control of the citrus PAL promoter, in the binary vector pCambia 2201

transferred from *E. coli* DH5 α to *A. tumefaciens* GV2260 by triparental mating for the transformation of *N. tabacum* cv. Samsun leaf disks, using the same procedure previously described (Chapter 4).

Shoots that grew from leaf disks in kanamycin selection medium were transferred to rooting medium and, subsequently, to soil. Leaf pieces were cut from ten regenerated plants and assayed for GUS activity. Surprisingly, none of the plants tested positive, which prompted more laborious PCR tests. Seeds from each one of the ten kanamycin-resistant, regenerated plants were collected and germinated in rooting medium supplemented with kanamycin 50 $\mu\text{g/ml}$. Seeds from the T-0 lines 6, 7, 12, 13, and 14 germinated on the selective medium and were transferred to soil. A total of 12 plants were obtained (three plants from each of the lines 6 and 12, and two plants from each of the lines 7, 13, and 14). These plants were tested for GUS activity and checked by PCR for the presence of the GUS gene (using primers CN216 5'-CAACGAAGTGAAGTGGCAG-3' and CN217 5'-CATCACCAGCTTGGGTG-3', that amplify a 817 bp fragment) and the cecropin gene (using the oligos CEC1 and CEC4, that amplify the 211 bp long cecropin gene). All plants again tested negative in GUS assays, but the GUS gene could be detected by PCR in all plants except 6.1, and the cecropin gene could be clearly detected in seven plants (7.1, 7.2, 12.2, 12.3, 13.1, 14.1, and 14.2) (Figures 5-6 and 5-7). The plants were kept in the greenhouse and analyzed later for cecropin gene expression by Northern blotting.

RNA was extracted from 0.5 g of leaf midribs using Trizol (Gibco-BRL), according to manufacturer's instructions. Approximately 8 μg of RNA from each of the transgenic plants was incubated for 10 min at 65°C in denaturation buffer (50% formamide, 3 M formaldehyde, 20 $\mu\text{g/ml}$ ethidium bromide, 0.1% bromophenol blue) and run at 40V for 3 h in a 1.2% agarose gel in TAE buffer. The gel was blotted to a nylon membrane using a downward capillary setting with 10x SSC (1.5 M NaCl, 0.15 M Na citrate, pH 7.0) as transferring buffer. The RNA was cross-linked to the membrane using a UV cross-linker (Stratagene). Hybridization was performed according to the manufacturer's instructions for the DIG RNA kit (Roche). Pre-hybridization was

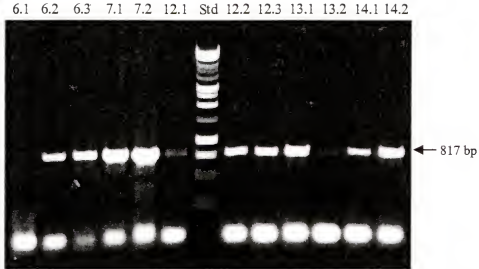


Figure 5-6. Detection of the GUS gene in T-1 transgenic tobacco plants. Std = 1 Kb ladder

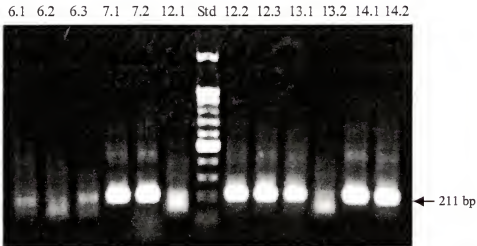


Figure 5-7. Detection of the cecropin gene in T-1 transgenic tobacco plants. Std = 100 bp ladder

done at 68°C for 1 h in a solution that consisted of 50% formamide, 5x SSC, 2% blocking reagent, 0.1% N-lauroylsarcosine, 0.02% SDS. Digoxigenin-labeled antisense RNA probe for the cecropin gene was transcribed from the CEC/pGEM-T plasmid, using T7 RNA polymerase and dNTPs mix with a 2:1 ratio of unlabeled:labeled UTP. Hybridization was performed with 100 ng/ml of the probe in pre-hybridization solution, overnight at 68°C. The membrane was washed twice for 15 min with 2x SSC, 0.1% SDS, at room temperature, and twice for 15 min with 0.1x

SSC, 0.1% SDS, at 68°C. Blocking was done with 1% blocking reagent in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) for 1 h at room temperature, and incubation with anti-DIG-alkaline phosphatase conjugate diluted 1:15,000 in blocking solution, at room temperature for 30 min. After washing twice for 15 min in maleic acid buffer with 0.3% (w/v) Tween 20, the membrane was developed with the chemiluminescent substrate CDP-Star, and the image captured with a Fluor-S MultiImager (BioRad).

Figure 5-8 shows a picture of the gel before transfer and of the membrane after hybridization. Cecropin mRNA was detected in the transgenic plants 6.2, 6.3, 13.1, and 13.2. The size of the detected mRNA was between 500-600 bases, which is in accordance with the size expected for the 189 bases long cecropin gene plus ~170 bases of the 5' UTR from the citrus PAL promoter and ~150 bases of the 3' UTR from the CaMV 35S terminator plus the poly-A tail.

A severe phenotypic alteration was observed in the T-1 progeny of transgenic lines 12 and 13. After apparently normal growth in the greenhouse for 1 month, these plants started to show a reduction in growth rate and alterations in leaf morphology. The leaves maintained an upright position and the plants senesced earlier than the descendants from line 14, which did not show phenotypic alteration (Figure 5-9). Progeny from lines 6 and 7 showed the same alteration but it appeared later and to a lesser extent.

Table 5-1 shows a summary of the results obtained with the T-1 transgenic plants. The correlation between cecropin gene expression and the phenotypic alteration is not clear. None of the tobacco plants transformed with an empty vector showed abnormalities, only those receiving the cecropin gene did. However, although cecropin expression could be detected in the severely affected plants 13.1 and 13.2, cecropin expression was not detected in the equally affected plants 12.1, 12.2, and 12.3.

There are few reports of deleterious effects of cationic antimicrobial peptide expression in transgenic plants. Okamoto *et al.* (1998) reported that transgenic tobacco plants expressing a sarcotoxin-GUS fusion protein were shorter than control ones and had thicker leaves with

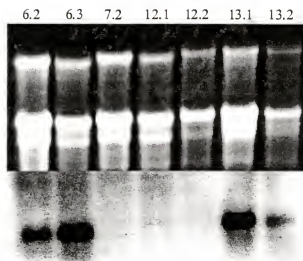


Figure 5-8. Northern blot analysis of the transgenic tobacco plants for expression of the cecropin gene. Upper picture shows agarose gel before blotting and lower picture shows the bands obtained after hybridization with the cecropin gene-specific probe.



Figure 5-9. Phenotype alteration in some of the transgenic plants. The picture shows, from left to right, transgenic lines 12.2, 12.3, 13.1 and 14.1. Among these, cecropin gene expression could be detected only in plant 13.1.

Plant	Phenotype ¹	Cecropin gene expression ²	GUS PCR ³	Cecropin PCR ⁴
6.1	Abnormal +	-	-	-
6.2	Abnormal +	+	+	-
6.3	Abnormal +	+	+	-
7.1	Abnormal +	-	+	+
7.2	Abnormal +	-	+	+
12.1	Abnormal ++	-	+	-
12.2	Abnormal ++	-	+	+
12.3	Abnormal ++	-	+	+
13.1	Abnormal ++	+	+	+
13.2	Abnormal ++	+	+	-
14.1	Normal	-	+	+
14.2	Normal	-	+	+

Table 5-1. Summary of the results obtained with the T-1 transgenic plants. (1) Phenotype as compared to plants transformed with empty vector (+ = reduction in plant size, ++ = severe reduction in plant size). (2) Detection of cecropin gene expression by Northern-blot. (3) Detection of GUS gene by PCR. (4) Detection of cecropin gene by PCR.

abnormal shape. The altered phenotype was observed in transgenic plants that had been transformed with different constructs having the fusion protein gene either with or without a signal peptide coding sequence. These authors showed that the signal peptide was not efficient for secretion of the sarcotoxin-GUS fusion protein, which accumulated in the cell cytoplasm. Detection of electrolyte leakage in their abnormal plants indicated damage of cell membranes. Transgenic lines expressing sarcotoxin with the signal peptide, but not fused to GUS, were efficient in the secretion of the protein and did not show abnormal phenotype. The altered phenotype described by these authors has some resemblance to what has been observed in the abnormal plants of the present study. It is possible that the signal peptide was not properly processed in the transgenic tobacco plants or that the high levels of gene expression overwhelmed the secretion machinery, in either case causing cecropin accumulation in the cytoplasm. In pear plants transgenic for the lytic peptide attacin, growth impairment has also been associated with

higher expression levels of the transgene that lacked a signal peptide coding sequence (Reynold et al., 1999).

Whether expression of the cecropin gene is or is not the cause of the altered phenotype cannot be answered by the analysis of the present results. A larger number of transgenic lines will have to be produced and analyzed to allow a better understanding of this phenomenon.

The limited number of transgenic plants produced and the drastic reduced growth observed in most of them did not allow realization of the *X. fastidiosa* inoculation assays. Such assays would be difficult to interpret using plants that show impaired growth without any pathogen challenge. It is possible to conduct growth studies of *X. fastidiosa* in planta, but such studies require long time periods to conduct, which were beyond the scope of the present study.

Transgenic apple and pear plants expressing an attacin gene have been shown to significantly reduce disease severity caused by *Erwinia amylovora* (Norelli et al., 1999; Reynold et al., 1999). Attacin is another cationic antimicrobial peptide produced by insects but its larger size (~20 kDa) may confer higher stability to this molecule. The purpose of using a cecropin gene was to test the hypothesis that expressing an antibacterial compound in the vicinity of xylem vessels can prevent systemic infection of the plant by *X. fastidiosa*. Since the sensitivity of the bacterium to cecropin A was demonstrated, expression of a gene coding for this type of peptide in a plant system would constitute an ideal tool to test the initial hypothesis that an inhibitory compound can be created and delivered into the xylem. Expressing a gene in the xylem parenchyma does not guarantee that its product will be transported to the xylem vessel lumen.

Despite of the phenotypic alteration observed in some of the transgenic tobacco plants expressing the cecropin gene, a larger number of plants will be produced in order to perform the inoculations with *X. fastidiosa* and to check for antibacterial activity in the sap. These plants will be obtained by germinating seeds from the T-0 and T-1 lines. New constructs with the cecropin gene under control of either the *Arabidopsis* PAL promoter or the CaMV 35S promoter, have already been produced and will be included in these experiments. Analysis of these plants will

allow the comparison of the effects of cecropin expression under control of three different promoters. The sweet orange PAL promoter drives gene expression preferentially, but not exclusively, in the xylem, the *Arabidopsis* PAL promoter is highly specific for the xylem, and the CaMV 35S promoter is not tissue-specific.

CHAPTER 6

COMPARISON BETWEEN CVC AND PD STRAINS OF *X. fastidiosa* THROUGH SUPPRESSION SUBTRACTIVE HYBRIDIZATION

With the completion of the genome sequencing of a CVC strain of *X. fastidiosa*, we start to understand the mechanisms that allow this pathogen to survive in a nutritionally poor environment such as the xylem sap of plants. Although strategies commonly employed by other Gram negative bacterial pathogens are not encoded by the *X. fastidiosa* genome, i.e. the type III secretion system and *avr/ptb* genes, other genes that have an obvious role in the pathogenicity could be found. Cell wall-degrading enzymes (endo-1,4- β -glucanases and an endo-polygalacturonase), adhesion-related surface proteins, exopolysaccharide synthesis, and a plethora of iron-acquisition mechanisms were found among the more than 2,700 genes of this bacterium.

What has not been answered by the sequencing of the CVC strain of *X. fastidiosa* is how host-specificity is controlled in this pathogen. The absence of homology to known *avr* genes eliminates the possibility that a common host range-determining mechanism of other Gram-negative pathogens is involved in this pathosystem.

A traditional way of finding host range-determining factors is to mobilize a genomic library from a donor strain to a recipient one and screen the latter for altered behavior in its original host plant. The lack of a fast pathogenicity assay to screen *X. fastidiosa* clones and, also, the lack of an efficient transformation or conjugation system preclude the use of this type of experiment for this pathogen.

It becomes clear that to answer these questions will require genetic studies comparing different strains of *X. fastidiosa*. Among the differences found, and having the complete sequence

of the CVC strain as a context to judge the relevance of these differences, it would be possible to select candidate genes involved in the host range determination.

A simplified subtractive hybridization procedure, called suppression subtractive hybridization (SSH), was developed by Diatchenko *et al.* (1996) to isolate genes that are differentially represented in two cDNA populations. Because bacterial genomes are small enough and are even less complex than many eukaryotic cDNA populations, SSH could be applied to find genetic differences between closely related bacterial strains (Akopyants *et al.*, 1998; Bogush *et al.*, 1999).

Figure 6-1 schematically represents the SSH procedure. Initially, genomic DNA from a tester and a driver strain is digested with a four-base-cutter enzyme. The digested tester DNA is split in two samples and ligated to different adaptors (1 and 2R). The first hybridization is performed separately for both adaptor-ligated tester DNA samples with an excess of driver DNA, generating four types of molecules (a = single-stranded DNA enriched for tester-specific sequences, b = self-annealed tester DNA, c = hybrid tester-driver molecules comprising most of the non-specific DNA, d = single-stranded and self-annealed driver DNA). In this hybridization step, the concentration of fragments present in single and multiple copies in the bacterial genome is equalized among the type "a" molecules, because more abundant sequences reanneal faster according to the second-order kinetics of hybridization. A second hybridization step is performed with fresh denatured driver DNA and both samples of non-denatured adaptor-ligated tester DNA from the previous step. Besides the four types of molecules already described, a new type "e" molecule will be formed, enriched for tester-specific fragments and having different adaptors at their 5' ends. The molecules are subject to a "fill in" step followed by PCR using a primer complementary to sequences common to both adaptors. Molecules "a" and "d" will not be amplified because they miss primer-binding sites. Most type "b" molecules form a panhandle-like structure, because of the complementary sequences at both ends, that prevents amplification (the suppression effect). Molecules type "c" will be amplified linearly because they have only one

primer-binding site. Only molecules of the type “e”, having two different adaptors at their ends, will be exponentially amplified. A second PCR is performed, using nested primers specific for each of the adaptors, that will further reduce any background PCR products and enrich for tester-specific sequences. The population of PCR products obtained is cloned in a “T/A” type of vector and a mini-library is constructed. This library can be screened by hybridization with labeled tester and driver whole DNA and confirm the tester-specific clones.

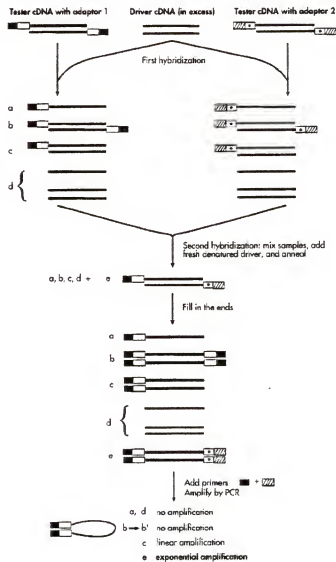


Figure 6-1. Schematic representation of the SSH procedure (from Diatchenko *et al.*, 1996)

A subtraction experiment was performed using CVC DNA as tester and PD DNA as driver. The procedure was performed according to the instructions of the commercially available SSH kit (PCR-Select Bacterial Genome Subtraction Kit - Clontech Laboratories, Inc.). Two micrograms of genomic DNA from each strain was digested with 15 U of *RsaI* restriction enzyme. About 240 ng of the digested CVC DNA sample was split in two and ligated to adaptors 1A and 2R (Figure 6-2). Approximately 600 ng of the digested PD DNA (driver) was added to each adaptor-ligated tester sample, and both mixtures were denatured at 98°C for 1.5 min and hybridized at 63°C for 1.5 h. A second hybridization was performed by adding 300 ng of freshly denatured driver DNA, mixing both tester samples from the previous hybridization, and incubating overnight at 63°C. The first PCR reaction was performed using Primer 1 and the following program: initial incubation at 72°C for 2 min ("fill in" step), denaturation at 94°C for 25 s and 25 cycles of 94°C/10 s – 66°C/30 s – 72°C/1.5 min. A tube temperature controlled thermocycler was employed (Hybaid – PCR Express). The first PCR mix was diluted 1:40 and submitted to the second PCR using nested primers 1 and 2R, and the following program: denaturation at 94°C for 25 s and 12 cycles of 94°C/10 s – 68°C/30 s – 72°C/1.5 min. The product of the second PCR reaction was submitted to purification using a QIAquick PCR purification kit (Qiagen) and ligated to a pGEM-T Easy vector (Promega).

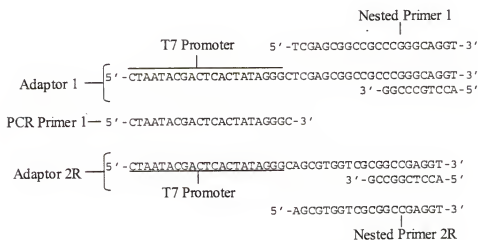


Figure 6-2. Sequences of the adaptors and primers employed for the SSH.

The colonies obtained after transformation of *E. coli* DH5 α were transferred to a master plate and kept at 4°C. During the process of picking colonies from the transformation plate and transferring to the master plate, the toothpicks used to streak each colony were agitated in 50 μ l of an extraction buffer (10 mM Tris, 2 mM EDTA, 0.1 % Triton X-100, pH 8.0) in microfuge tubes. These samples were heated in boiling water for 10 min, cooled on ice for 5 min, spun at maximum speed for 10 min, and 1 μ l of the supernatant used as template to PCR-screen the mini-library. The screening reaction was performed with vector-based primers T7 and SP6 and using the following program: initial denaturation at 94°C/2 min, 40 cycles of 94°C/20 s – 56°C/30 s – 72°C/1 min, and final extension at 72°C/5 min. A 6 μ l aliquot of each PCR reaction was loaded on a 1.5 % agarose gel. A total of 95 clones were screened and Figure 6-3 shows the results for samples 51 through 98. The inserts size were in the range from 200 to 1200 bp, with the majority within 300 to 500 bp (the use of the vector based primers T7 and SP6 add 175 bp to the PCR product)

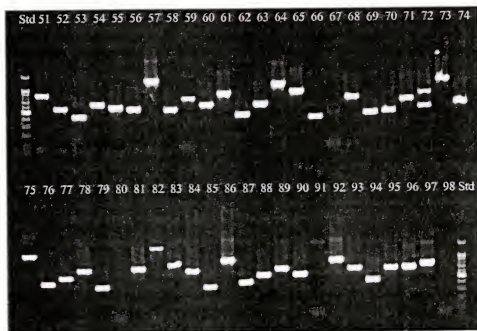


Figure 6-3. PCR-screening of putative CVC-specific clones 51 through 98, obtained after SSH. Std = 100 bp ladder (Promega)

For confirmation of the CVC-specificity of each clone, the PCR products were hybridized to total DNA, from both CVC and PD, labeled with digoxigenin. The labeling was performed by random priming using hexamers, Klenow fragment of the DNA polymerase, and dNTPs mix containing digoxigenin-labeled dUTP (2:1 unlabeled-labeled ratio). The template consisted of 1 μ g of RsaI-digested CVC and PD genomic DNA.

One microliter of the PCR products obtained from each clone of the CVC-specific mini-library was denatured by heating to 98°C for 2 min and spotted on duplicated nylon membranes. One of them was hybridized to the digoxigenin-labeled DNA from CVC and the other to the labeled PD DNA. Hybridizations were performed at 65°C as well as the stringent washes with 0.5X SSC. After incubation with the alkaline phosphatase-anti DIG conjugate, the membranes were developed with the chemiluminescent substrate CDP-Star and exposed to X-ray films. Clones that hybridized to the CVC probe and gave very weak or no signal with the PD probe were considered CVC-specific. Figure 6-4 shows the results for the clones 1 through 47 and 51 through 98. In order to facilitate the comparison of the hybridization signals, the two X-ray films corresponding to membranes incubated with the CVC and the PD probes were superimposed, aligning side by side the spots. Therefore, in Figure 6-4, for each pair of spots, the one to the right was obtained after hybridization with the CVC probe and the one to the left, with the PD probe. Spots identified as "48" are ~100 ng of total DNA from CVC (right) and PD (left).

The CVC-specificity of some of the clones was further confirmed by Southern blot analyses. Clones CVC-2, CVC-10, CVC-25, and CVC-29 were digoxigenin-labeled by PCR and hybridized against genomic DNA from CVC and PD digested with EcoRI. Figure 6-5 shows the results of these hybridizations. With the exception of clone CVC-25, which gave a weak signal with PD DNA, the Southern blot confirmed the specificity of the clones.

The 18 clones indicated by a dotted box in Figure 6-4 (2, 10, 16, 25, 29, 32, 40, 46, 51, 70, 75, 77, 83, 86, 88, 89, 90, and 95) were sequenced. Using BLAST against the *X. fastidiosa*

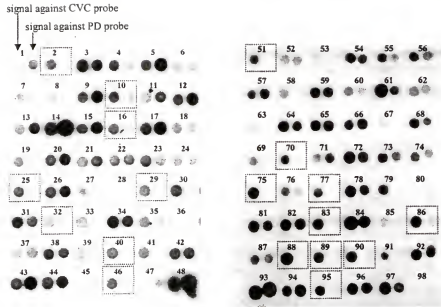


Figure 6-4. Dot blot hybridization of PCR products from the CVC-specific mini-library, against digoxigenin-labeled CVC DNA (right spot) and PD DNA (left spot). Dotted boxes indicate clones that were selected for sequencing.

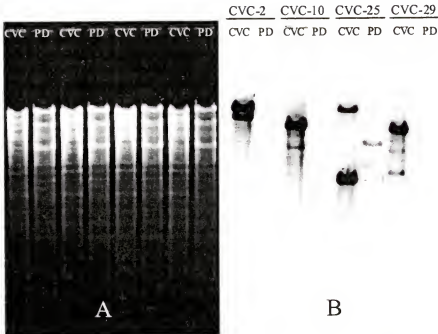


Figure 6-5. Confirmation of the CVC-specificity of the clones CVC-2, CVC-10, CVC-25, and CVC-29, by Southern blot. A – gel before the transfer. B – Strips from the membrane, hybridized to digoxigenin-labeled probes corresponding to each of the clones indicated on the top.

genome, it was found that seven of these clones (2, 32, 46, 51, 70, 77, and 88) corresponded to different genes of a 51 kb megaplasmid of the CVC strain, which was not present in the PD strain used in the present study. The other 11 clones corresponded to chromosomal genes and, with the exception of the identical clones 89 and 95, all of them contained distinct sequences.

Clones CVC-10 and CVC-75 correspond to sequences with homology to different genes of the filamentous bacteriophage Pf3 of *Pseudomonas aeruginosa*. Interestingly, homology to sequences of the same phage had already been reported for the plasmid XF868 (Pooler *et al.*, 1997). This plasmid was found in a Mulberry strain of *X. fastidiosa*, and Southern analysis indicated that it was also present in the Periwinkle strain, but not in 5 different isolates of CVC tested in that study. Accordingly, clones 10 and 75 hit bacteriophage Pf3 genes that are not present in the plasmid XF868.

Clone CVC-16 shows homology to the 5' end of a *traF* gene from the *E. coli* Inc P plasmid R751. The *X. fastidiosa traF* homologue is located in a cluster containing several conjugal transfer-related "*trb*" genes. It probably corresponds to a plasmid that has incorporated itself into the chromosome.

Clone CVC-25 has homology to the gene that codes for the hypothetical protein Rv2515 (GenBank Z95556) of *Mycobacterium tuberculosis*.

Clone CVC-29 has a weak resemblance to a replication protein from the plasmid pHT926 from *Brevibacillus borstelensis*. The *X. fastidiosa* gene corresponding to this clone is located next to the bacteriophage Pf3 homologue that corresponds to clone 10.

Clone CVC-40 has homology to the C-terminus of a putative DNA primase of the *Lactobacillus casei* bacteriophage A2. The location in the *X. fastidiosa* chromosome is within one of the 4 large prophages found in the genome of this bacterium.

Clone CVC-83 has homology to the gene *pqaA* from *Salmonella typhi*, which is regulated by the two-component system PhoP/Q but whose function is unknown.

The identical clones CVC-89 and CVC-95 correspond to the 3' end of a *X. fastidiosa* gene that has similarity to the rolling circle replication protein A gene from the *E. coli* bacteriophage 186. This *X. fastidiosa* gene is located in the same cluster as those that corresponded to clones 10, 29, and 75.

Clone CVC-90 corresponded to the 3' end of an ORF that does not show homology to any sequence in the GenBank.

These results clearly demonstrate that SSH is a powerful tool for cloning DNA sequences that are differentially represented in two closely related bacterial isolates. The simplicity of the procedure allows the whole process to be completed in no more than a week. That from 18 clones sequenced, only two were identical, indicates the complexity of the mini-library. Therefore, many more clones could be sequenced before reaching excessive redundancy.

It was found that four clones (10, 29, 75, and 89/95) represented fragments of genes located in the same cluster. It would be interesting to find where are the boundaries of this cluster, that enclose sequences specific for the CVC strain. Using genes flanking the ones that have already been shown to be specific for the CVC strain, as probes in Southern blots against PD DNA could provide the answer. Probes progressively farther, in both directions, from the CVC- specific region would be used until reaching sequences present in both strains. The same procedure could be used for the other clones that are scattered throughout the bacterial chromosome, to find out if they are within a cluster of differentially represented genes.

Because a limited number of clones were sequenced, formulating a hypothesis to explain the differences in host-specificity between the CVC and PD strains is speculative. Nevertheless, the presence of many phage-related sequences in the subtracted library suggests that these elements are important components of the variability among *X. fastidiosa* strains. Bacteriophage as mediators of horizontal gene transfer is a frequent phenomenon among bacteria (Hendrix *et al.*, 1999) and the results of the present study suggest that it may also have happened in the evolution of *X. fastidiosa* strains. Bacteriophage could act positively, by bringing new and advantageous

genetic information to its new host, or negatively, by interrupting resident genes that would cause rejection of the bacterium in a new prospective host plant species.

The absence of the megaplasmid in the PD strain was not anticipated because preliminary studies employing PCR with primers based on the sequence of a conjugation-related gene (*trbF*), and thought to be specific for the megaplasmid, indicated that it was present. It turned out that a copy of that gene was also present in the chromosomal DNA. Sequence analysis of the megaplasmid does not show evidence that it is involved in pathogenicity. Whether this plasmid, or related ones, are present in other *X. fastidiosa* strains is not known.

It is noteworthy that the reciprocal subtraction, using CVC DNA as driver and PD DNA as tester, was attempted without success. Of 47 clones analyzed, none revealed to be specific for the PD strain. Further experiments are necessary to determine whether it was due to technical problems or because the evolution of the CVC strain occurred through addition of new genes to the basic set of the PD strain, in which case the reciprocal subtraction would not render PD-specific fragments.

CHAPTER 7

CONCLUSIONS

The possibility of using tobacco as model for the study of *X. fastidiosa*-host plant interactions and for the development of transgenic resistance against this pathogen can considerably shorten experimental time, and will enable the execution of research projects otherwise unviable if original perennial hosts had to be used.

The picture that arises from the complete sequencing of the *X. fastidiosa* genome is of an semi-specialized endophyte, without highly evolved pathogenicity mechanisms such as type III secretion system and *avr/pt* genes, that encodes in a relatively small chromosome all the genes required for survival in a nutritionally poor environment and for hitch-hiking in a very efficient vector. Our inability to identify among the more than 2,700 genes, a few ones that would constitute a key pathogenicity mechanism, suggests that there may not be a silver bullet able to control *X. fastidiosa* and that multiple weapons aimed at multiple targets will have to be developed.

Development of resistance against *X. fastidiosa* through genetic manipulation of the host requires expression of the transgene in the proper tissues. The possibility of using a native promoter to drive transgene expression in the xylem was investigated in the present study. Partial success was attained in the cloning of a xylem-specific promoter from sweet orange. The “leaky” behavior of the citrus PAL promoter in tissues other than the xylem will have to be further investigated. It may have been an abnormality due to tobacco not providing the requisites for a proper manifestation of the citrus promoter, or a consequence of using a fragment of it that was not long enough to confer xylem-specificity. The approach of using Inverse-PCR proved

successful and could be employed for the cloning of other native promoters with different characteristics in terms of tissue- or developmental stage-specificity, and responsiveness to endogenous or exogenous factors.

A modified cecropin gene was successfully synthesized and expressed in transgenic tobacco. Growth impairment was observed in some of the transgenic lines produced, and the possibility that expression of cecropin in xylem tissues is toxic to the plant will have to be further investigated.

The use of synthetic genes will probably increase in the near future. Besides allowing the optimization of codon composition for a particular host and circumventing lengthy cloning processes, a better understanding of protein domains and motifs will make possible the construction of “designer genes”. The success in synthesizing a small gene and the continuous reduction of oligonucleotide prices encourage employing the same strategy for longer genes.

The availability of a complete genome sequence for one strain of a bacterial pathogen opens the opportunity to understand the evolution of the species as a whole. The mechanisms of genetic change and how they create new pathovars might be answered by finding the genetic differences between the sequenced strain and related ones. Suppression subtractive hybridization seems to be a very simple and efficient method to compare the genomes of CVC and PD strains of *X. fastidiosa*. The limited number of clones analyzed in the present study allows a few insights about the differences between the two *X. fastidiosa* strains, but a continuation of the strategy started here should provide important information towards a broader view of the evolution of this plant pathogen.

REFERENCES

- Akopyants, N. S., Fradkov, A., Diatchenko, L., Hill, J. E., Siebert, P. D., Lukyanov, S. A., Sverdlov, E. D., and Berg, D. E. (1998). PCR-based subtractive hybridization and differences in gene content among strains of *Helicobacter pylori*. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 13108-13113.
- Allefs, S., Florack, D. E. A., Hoogendoorn, C., and Stiekema, W. J. (1995). *Erwinia* soft-rot resistance of potato cultivars transformed with a gene construct coding for antimicrobial peptide cecropin-B is not altered. *American Potato Journal* **72**, 437-445.
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1997). *Short Protocols in Molecular Biology*, 3rd edition. New York: John Wiley and Sons Inc.
- Bent, A. F., and Yu, I. C. (1999). Applications of molecular biology to plant disease and insect resistance. In *Advances in Agronomy*, Vol 66, pp. 251-298. San Diego: Academic Press Inc.
- Bevan, M., Shufflebottom, D., Edwards, K., Jefferson, R., and Schuch, W. (1989). Tissue-specific and cell-specific activity of a phenylalanine ammonia-lyase promoter in transgenic plants. *Embo Journal* **8**, 1899-1906.
- Bogush, M. L., Velikodvorskaya, T. V., Lebedev, Y. B., Nikolaev, L. G., Lukyanov, S. A., Fradkov, A. F., Pliyev, B. K., Boichenko, M. N., Usatova, G. N., Vorobiev, A. A., Andersen, G. L., and Sverdlov, E. D. (1999). Identification and localization of differences between *Escherichia coli* and *Salmonella typhimurium* genomes by suppressive subtractive hybridization. *Molecular and General Genetics* **262**, 721-729.
- Carmona, M. J., Molina, A., Fernandez, J. A., Lopezfando, J. J., and Garciaolmedo, F. (1993). Expression of the alpha-thionin gene from barley in tobacco confers enhanced resistance to bacterial pathogens. *Plant Journal* **3**, 457-462.
- Chagas, C. M., Rossetti, V., and Beretta, M. J. G. (1992). Electron microscopy studies of a xylem-limited bacterium in sweet orange affected with citrus variegated chlorosis disease in Brazil. *Journal of Phytopathology* **134**, 306-312.
- Chang, C. J., Garnier, M., Zreik, L., Rossetti, V., and Bove, J. M. (1993). Culture and serological detection of the xylem-limited bacterium causing citrus variegated chlorosis and its identification as a strain of *Xylella fastidiosa*. *Current Microbiology* **27**, 137-142.
- Chang, C. J., Robacker, C. D., and Lane, R. P. (1990). Further evidence for the isolation of *Xylella fastidiosa* on nutrient agar from grapevines showing Pierce's disease symptoms. *Canadian Journal of Plant Pathology* **12**, 405-408.

- Chen, J., Chang, C. J., Jarret, R. L., and Gawel, N. (1992). Genetic variation among *Xylella fastidiosa* strains. *Phytopathology* **82**, 973-977.
- Chen, Y. D., and Chen, T. A. (1998). Expression of engineered antibodies in plants: A possible tool for spiropasma and phytoplasma disease control. *Phytopathology* **88**, 1367-1371.
- Costa, P. I., Franco, C. F., Miranda, V. S., Teixeira, D. C., and Hartung, J. S. (2000). Strains of *Xylella fastidiosa* rapidly distinguished by arbitrarily primed-PCR. *Current Microbiology* **40**, 279-282.
- Davis, M. J., French, W. J., and Schaad, N. W. (1981). Isolation and culture of the bacteria associated with phony peach disease and plum leaf scald. *Phytopathology* **71**, 869-870.
- Davis, M. J., Purcell, A. H., and Thomson, S. V. (1978). Pierce's disease of grapevines: isolation of the causal bacterium. *Science* **199**, 75-77.
- Davis, M. J., Thomson, S. V., and Purcell, A. H. (1980). Etiological role of the xylem-limited bacterium causing Pierce's disease in almond (*Prunus amygdalus*) leaf scorch. *Phytopathology* **70**, 472-475.
- Derrick, K. S., Lee, R. F., Brlansky, R. H., Timmer, L. W., Hewitt, B. G., and Barthe, G. A. (1990). Proteins associated with citrus blight. *Plant Disease* **74**, 168-170.
- Diatchenko, L., Lau, Y. F. C., Campbell, A. P., Chenchik, A., Moqadam, F., Huang, B., Lukyanov, S., Lukyanov, K., Gurskaya, N., Sverdlov, E. D., and Siebert, P. D. (1996). Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 6025-6030.
- Di Donato, A., Denigris, M., Russo, N., Dibiase, S., and Dalessio, G. (1993). A method for synthesizing genes and cDNAs by the polymerase chain-reaction. *Analytical Biochemistry* **212**, 291-293.
- During, K. (1996). Genetic engineering for resistance to bacteria in transgenic plants by introduction of foreign genes. *Molecular Breeding* **2**, 297-305.
- During, K., Porsch, P., Fladung, M., and Lorz, H. (1993). Transgenic potato plants resistant to the phytopathogenic bacterium *Erwinia carotovora*. *Plant Journal* **3**, 587-598.
- Fry, S. M., Milholland, R. D., and Huang, P. Y. (1990). Isolation and growth of strains of *Xylella fastidiosa* from infected grapevines on nutrient agar media. *Plant Disease* **74**, 522-524.
- Gabriel, D. W. (1999). Why do pathogens carry avirulence genes? *Physiological and Molecular Plant Pathology* **55**, 205-214.
- Giacomin, L. T., and Szalay, A. A. (1996). Expression of a PAL I promoter luciferase gene fusion in *Arabidopsis thaliana* in response to infection by phytopathogenic bacteria. *Plant Science* **116**, 59-72.
- Goheen, A. C., Nyland, G., and Lowe, S. K. (1973). Association of a rickettsialike organism with Pierce's disease of grapevines and alfalfa dwarf and heat therapy of the disease in grapevines. *Phytopathology* **63**, 341-345.

- Gray-Mitsumune, M., Molitor, E. K., Cukovic, D., Carlson, J. E., and Douglas, C. J. (1999). Developmentally regulated patterns of expression directed by poplar PAL promoters in transgenic tobacco and poplar. *Plant Molecular Biology* 39, 657-669.
- Grosser, J. W., and Gmitter, F. G. Jr. (1990). Protoplast fusion and citrus improvement. *Plant Breeding Reviews* 8, 339-374.
- Hancock, R. E. W., and Lehrer, R. (1998). Cationic peptides: A new source of antibiotics. *Trends in Biotechnology* 16, 82-88.
- Hendrix, R. W., Smith, M. C. M., Burns, R. N., Ford, M. E., and Hatfull, G. F. (1999). Evolutionary relationships among diverse bacteriophages and prophages: All the world's a phage. Proceedings of the National Academy of Sciences of the United States of America 96, 2192-2197.
- Hightower, R., Baden, C., Penzes, E., and Dunsmuir, P. (1994). The expression of cecropin peptide in transgenic tobacco does not confer resistance to *Pseudomonas syringae* pv *tabaci*. *Plant Cell Reports* 13, 295-299.
- Hopkins, D. L. (1989). *Xylella fastidiosa*: Xylem-limited bacterial pathogen of plants. *Annual Review of Phytopathology* 27, 271-290.
- Hopkins, D. L., and Mollenhauer, H. H. (1973). Rickettsia-like bacterium associated with Pierce's disease of grapes. *Science* 179, 298-300.
- Huang, Y., and McBeath, J. H. (1994). Bacterial induced activation of an arabidopsis phenylalanine ammonia-lyase promoter in transgenic tobacco plants. *Plant Science* 98, 25-35.
- Huang, Y., Nordeen, R. O., Di, M., Owens, L. D., and McBeath, J. H. (1997). Expression of an engineered cecropin gene cassette in transgenic tobacco plants confers disease resistance to *Pseudomonas syringae* pv *tabaci*. *Phytopathology* 87, 494-499.
- Kamper, S. M., French, W. J., and Dekloet, S. R. (1985). Genetic relationships of some fastidious xylem-limited bacteria. *International Journal of Systematic Bacteriology* 35, 185-188.
- Kawamata, S., Shimoharai, K., Imura, Y., Ozaki, M., Ichinose, Y., Shiraishi, T., Kunoh, H., and Yamada, T. (1997). Temporal and spatial pattern of expression of the pea phenylalanine ammonia-lyase gene1 promoter in transgenic tobacco. *Plant and Cell Physiology* 38, 792-803.
- Laranjeira, F. F., Pompeu Jr, J., Harakava, R., Figueredo J. O., Carvalho, S. A., and Coletta Filho, H. D. (1998). Citrus varieties and species host of *Xylella fastidiosa* under field conditions. *Fitopatologia Brasileira* 23, 147-154.
- Lee, R., Beretta M. J. G., Hartung, J. S., Hooker, M. E., and Derrick, K. S. (1993). Citrus variegated chlorosis: confirmation of a *Xylella fastidiosa* as the causal agent. *Summa Phytopathologica* 19, 123-125.
- Liang, X., Dron, M., Schmid, J., Dixon, R. A., and Lamb, C. J. (1989). Developmental and environmental regulation of a phenylalanine ammonia-lyase beta-glucuronidase gene fusion

- in transgenic tobacco plants. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 9284-9288.
- Lima, J. E. O., Miranda, V. S., Hartung, J. S., Brilansky, R. H., Coutinho, A., Roberto, S. R., and Carlos, E. F. (1998). Coffee leaf scorch bacterium: Axenic culture, pathogenicity, and comparison with *Xylella fastidiosa* of citrus. *Plant Disease* **82**, 94-97.
- Logemann, E., Parniske, M., and Hahlbrock, K. (1995). Modes of expression and common structural features of the complete phenylalanine ammonia-lyase gene family in parsley. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 5905-5909.
- Martemyanov, K. A., Spirin, A. S., and Gudkov, A. T. (1996). Synthesis, cloning and expression of genes for antibacterial peptides: cecropin, magainin and bombinin. *Biotechnology Letters* **18**, 1357-1362.
- Martin, C., and Paz-Ares, J. (1997). MYB transcription factors in plants. *Trends in Genetics* **13**, 67-73.
- Minsavage, G. V., Thompson, C. M., Hopkins, D. L., Leite, R. M. V. B. C., and Stall, R. E. (1994). Development of a polymerase chain reaction protocol for detection of *Xylella fastidiosa* in plant tissue. *Phytopathology* **84**, 456-461.
- Molina, A., and Garcia-Olmedo, F. (1997). Enhanced tolerance to bacterial pathogens caused by the transgenic expression of barley lipid transfer protein LTP2. *Plant Journal* **12**, 669-675.
- Nakajima, H., Muranaka, T., Ishige, F., Akutsu, K., and Oeda, K. (1997). Fungal and bacterial disease resistance in transgenic plants expressing human lysozyme. *Plant Cell Reports* **16**, 674-679.
- Nordeen, R. O., Sinden, S. L., Jaynes, J. M., and Owens, L. D. (1992). Activity of cecropin SB37 against protoplasts from several plant-species and their bacterial pathogens. *Plant Science* **82**, 101-107.
- Norelli, J. L., Borejsza-Wysocka, E. E., Reynoird, J.-P., and Aldwinckle, H. S. (1999). Transgenic Gala apple expressing attacin E has increased field resistance to *Erwinia amylovora* (fire blight). *Phytopathology* **89**, S56.
- Ochman, H., Gerber, A. S., and Hartl, D. L. (1988). Genetic applications of an inverse polymerase chain-reaction. *Genetics* **120**, 621-623.
- Ohl, S., Hedrick, S. A., Chory, J., and Lamb, C. J. (1990). Functional properties of a phenylalanine ammonia-lyase promoter from *Arabidopsis*. *Plant Cell* **2**, 837-848.
- Ohshima, M., Mitsuhashi, I., Okamoto, M., Sawano, S., Nishiyama, K., Kaku, H., Natori, S., and Ohashi, Y. (1999). Enhanced resistance to bacterial diseases of transgenic tobacco plants overexpressing sarcotoxin IA, a bactericidal peptide of insect. *Journal of Biochemistry* **125**, 431-435.
- Okamoto, M., Mitsuhashi, I., Ohshima, M., Natori, S., and Ohashi, Y. (1998). Enhanced expression of an antimicrobial peptide sarcotoxin IA by GUS fusion in transgenic tobacco plants. *Plant and Cell Physiology* **39**, 57-63.

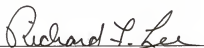
- Oldroyd, G. E. D., and Staskawicz, B. J. (1998). Genetically engineered broad-spectrum disease resistance in tomato. *Proceedings of the National Academy of Sciences of the United States of America* 95, 10300-10305.
- Owens, L. D., and Heutte, T. M. (1997). A single amino acid substitution in the antimicrobial defense protein cecropin B is associated with diminished degradation by leaf intercellular fluid. *Molecular Plant-Microbe Interactions* 10, 525-528.
- Pooler, M. R., and Hartung, J. S. (1995). Genetic relationships among strains of *Xylella fastidiosa* from RAPD-PCR data. *Current Microbiology* 31, 134-137.
- Purcell, A. H., and Finlay, A. (1979). Evidence for non-circulative transmission of Pierce's disease bacterium by sharpshooter leafhoppers. *Phytopathology* 69, 393-395.
- Purcell, A. H., and Hopkins, D. L. (1996). Fastidious xylem-limited bacterial plant pathogens. *Annual Review of Phytopathology* 34, 131-151.
- Purcell, A. H., and Saunders, S. R. (1999). Fate of Pierce's disease strains of *Xylella fastidiosa* in common riparian plants in California. *Plant-Disease* 83, 825-830.
- Reynold, J. P., Mourgues, F., Norelli, J., Aldwinckle, H. S., Brisset, M. N., and Chevreau, E. (1999). First evidence for improved resistance to fire blight in transgenic pear expressing the attacin E gene from *Hyalophora cecropia*. *Plant Science* 149, 23-31.
- Rosato, Y. B., Rodrigues Neto, J., Miranda, V. S., Carlos, E. F., and Manfio, G. P. (1998). Diversity of a *Xylella fastidiosa* population isolated from *Citrus sinensis* affected by citrus variegated chlorosis in Brazil. *Systematic and Applied Microbiology* 21, 593-598.
- Sablowski, R. W. M., Baulcombe, D. C., and Bevan, M. (1995). Expression of a flower-specific myb protein in leaf-cells using a viral vector causes ectopic activation of a target promoter. *Proceedings of the National Academy of Sciences of the United States of America* 92, 6901-6905.
- Sakuta, C., Oda, A., Yamakawa, S., and Satoh, S. (1998). Root-specific expression of genes for novel glycine-rich proteins cloned by use of an antiserum against xylem sap proteins of cucumber. *Plant and Cell Physiology* 39, 1330-1336.
- Sakuta, C., and Satoh, S. (1997). cDNA cloning of xylem sap proteins and the analysis of their expressions in cucumber plants. *Plant Physiology* 114, 1340-1340.
- Shufflebottom, D., Edwards, K., Schuch, W., and Bevan, M. (1993). Transcription of 2 members of a gene family encoding phenylalanine ammonia-lyase leads to remarkably different cell specificities and induction-patterns. *Plant Journal* 3, 835-845.
- Tang, X. Y., Xie, M. T., Kim, Y. J., Zhou, J. M., Klessig, D. F., and Martin, G. B. (1999). Overexpression of Pto activates defense responses and confers broad resistance. *Plant Cell* 11, 15-29.
- Triglia, T., Peterson, M. G., and Kemp, D. J. (1988). A procedure for invitro amplification of dna segments that lie outside the boundaries of known sequences. *Nucleic Acids Research* 16, 8186-8186

- Wells, J. M., Raju, B. C., Hung, H. Y., Weisburg, W. G., Mandelco Paul, L., and Brenner, D. J. (1987). *Xylella fastidiosa*, new genus new species Gram-negative, xylem-limited, fastidious plant bacteria related to *Xanthomonas* spp. *International Journal of Systematic Bacteriology* **37**, 136-143.
- Wells, J. M., Raju, B. C., Nyland, G., and Lowe, S. K. (1981a). Medium for isolation and growth of bacteria associated with plum leaf scald and phony peach diseases. *Applied and Environmental Microbiology* **42**, 357-363.
- Wells, J. M., Raju, B. C., Thompson, J. M., and Lowe, S. K. (1981b). Common etiology of phony peach and plum leaf scorch diseases. *Phytopathology* **71**, 912-912.
- Zhang, Z. Y., Coyne, D. P., Vidaver, A. K., and Mitra, A. (1998). Expression of human lactoferrin cDNA confers resistance to *Ralstonia solanacearum* in transgenic tobacco plants. *Phytopathology* **88**, 730-734.
- Zhang, L. H., Xu, J. L., and Birch, R. G. (1999). Engineered detoxification confers resistance against a pathogenic bacterium. *Nature Biotechnology* **17**, 1021-1024.
- Zhu, Q., Dabi, T., Beeche, A., Yamamoto, R., Lawton, M. A., and Lamb, C. (1995). Cloning and properties of a rice gene encoding phenylalanine ammonia-lyase. *Plant Molecular Biology* **29**, 535-550.

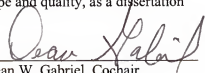
BIOGRAPHICAL SKETCH

Ricardo Harakava was born and raised in the city of São Paulo, Brazil. During his undergraduate studies in biological sciences at the University of São Paulo, he was invited to an internship at the Phytopathological Biochemistry lab at the Instituto Biológico (IB). This first contact with plant pathology triggered his interest on the subject of molecular plant-microbe interactions. After receiving a BS degree, he entered the masters program in agronomy at the University of São Paulo. In 1992, he defended his master's thesis on the study of the coffee leaf rust fungus, *Hemileia vastatrix*, using serological methods. In 1993 he was hired by IB as a research assistant, and he was promoted to full scientific researcher in 1997, just a few months after starting his PhD program in the Plant Pathology Department of the University of Florida.

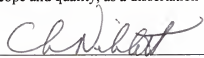
I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.


Richard F. Lee, Chair
Professor of Plant Pathology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.


Dean W. Gabriel, Cochair
Professor of Plant Pathology

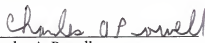
I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.


Charles L. Niblett
Professor of Plant Pathology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.


Kenneth S. Derrick
Professor of Plant Pathology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.


Charles A. Powell
Professor of Plant Pathology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



Gloria A. Moore

Professor of Horticultural Sciences

This dissertation was submitted to the Graduate Faculty of the College of Agricultural and Life Sciences and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August 2000



Dean, College of Agricultural and
Life Sciences

Dean, Graduate School